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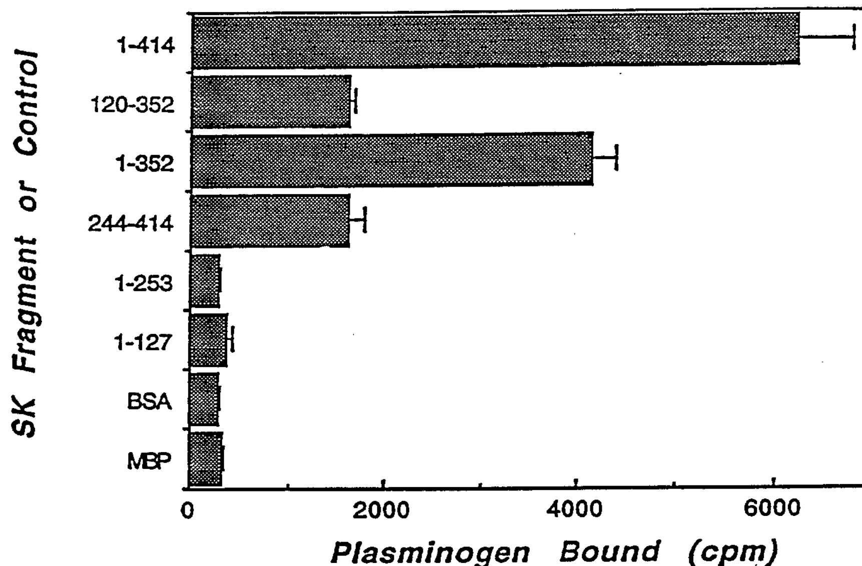

(57) Abstract

The present invention is directed to nucleic acids which encode polypeptides that bind with specificity to plasminogen and which correspond to regions of streptokinase. The invention is also directed to vectors and hosts which express such nucleic acids and to the polypeptides themselves. The binding of various purified, cleaved recombinant streptokinase fragments to ^{125}I-plasminogen is shown. In addition, the invention is directed to the use of the claimed polypeptides in assays which detect the presence of plasminogen. Streptokinase fragments which retain their ability to activate plasminogen may be used therapeutically.

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Peptides Specifically Binding to Plasminogen And the DNA Encoding Such Peptides

Statement of Government Rights in the Invention

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

The invention is in the field of immunology and molecular biology as related to streptokinase and fragments of streptokinase.

Cross-Reference to Related Applications

This application is a continuation-in-part of United States Patent Application No. 07/956,692 (filed on October 5, 1992) which is incorporated herein by reference.

Background of the Invention

The plasminogen activator streptokinase (hereinafter "SK") is widely used to dissolve blood clots that cause heart attacks. SK is derived from various streptococcus species and differs significantly from the naturally occurring human plasminogen activators urokinase and tissue plasminogen activator (tPA). Urokinase and tPA are serine proteases that cleave a peptide bond in plasminogen to convert it to plasmin, the active enzyme that degrades fibrin. Unlike these human plasminogen activators, SK has no intrinsic enzymatic activity (reviewed in Reddy, K. N., *Enzyme 40*:79-89 (1988)).

The mechanism by which SK and plasminogen form a functional plasminogen activator is not known. After completing their analysis of the primary structure of SK, Jackson and Tang (1982) noted that the 245 amino-terminal residues of SK were homologous with sequences from various serine proteases (*Biochemistry 21*:6620-6625 (1982)). They speculated that SK had

evolved from a serine protease but had lost enzymatic activity when a histidine from its putative active site had been mutated to a glycine.

Similarly, Taylor and Beisswenger (1973) proposed that SK contained the active site of the plasminogen activator (*J. Biol. Chem. 248*:1127-11134 (1973)). They observed that SK was proteolytically modified after binding to plasminogen and reported that this modified SK could be isolated and shown to be active. However, their findings were later challenged by Brockway and Castellino (*Biochemistry 13*:2063-2070 (1974)) and have not been confirmed.

The most widely accepted theory of SK's action is that it functions as an allosteric modifier of plasminogen. Two different mechanistic models of this theory have been proposed. One model proposes that SK binds tightly to plasminogen, alters its conformation, and converts it to a plasminogen activator. The modified plasminogen in the SK-plasminogen complex then cleaves the Arg_{560}-Val bond in another plasminogen molecule to form plasmin (Reddy, and Markus, *J. Biol. Chem. 247*:1683-1691 (1972); Schick and Castellino, *Biochemistry 12*:4315-4321 (1973)).

The other mechanistic model, proposed by Kosow, also postulates that SK functions as a catalytic allosteric effector of plasminogen, but by a different mechanism (Kosow, *Biochemistry 14*:4459-4465 (1975)). In this model, SK binds to plasminogen and changes its conformation. This produces an internal active site in plasminogen that "auto"-cleaves an internal bond to produce plasmin. According to this model, SK then dissociates to bind to another plasminogen molecule to repeat the process.

Both of the proposed models postulate that SK acts merely as an allosteric modifier of plasminogen, although they differ in the stability they ascribe to the SK-plasminogen complex and as to whether plasminogen activates itself or other molecules.

Despite speculation concerning the mechanism by which SK exerts its therapeutic effect, the prior art has not defined which regions of the protein bind to plasminogen. The invention disclosed herein relates to peptides which

have amino acid sequences corresponding to specific regions of SK and which bind to plasminogen.

Summary

The invention is directed, *inter alia*, to nucleic acids encoding portions of the streptokinase protein which are capable of binding to plasminogen. Included within the invention are all such nucleic acids encoding polypeptides having sequences corresponding to amino acids 14 to 414 of streptokinase except for the polypeptide consisting of amino acids 60 to 414. Preferably the encoded protein includes the amino acid sequence corresponding to amino acids 244 to 352 of streptokinase. The most preferred embodiments are those nucleic acids encoding proteins with sequences corresponding to amino acids 1 to 352; 120 to 352; 244 to 414; 244 to 352 or 14 to 414 of streptokinase.

The present invention is also directed to vectors and hosts which express the above nucleic acids and to the proteins themselves. The proteins may be used in assays designed to detect plasminogen in biological samples. Proteins which retain their ability to activate plasminogen may be used as a fibrinolytic in the same way that intact streptokinase has been used for decades.

In addition, the invention is directed to a method for assaying streptokinase fragments for antigenicity. This is accomplished by examining the ability of monoclonal antibodies to distinct epitopes of streptokinase to bind peptides with amino acid sequences corresponding to different regions of the streptokinase molecule.

The streptokinase fragment most preferred as a fibrinolytic agent is SK_{14-414}. This fragments exhibits reduced antigenicity relative to intact streptokinase but retains the ability of the intact protein to activate plasminogen.

Brief Description of the Drawings

Figure 1: Competitive epitope binding assays for six different categorical MAbs. Wells of a microtiter plate were coated with the purified MAb indicated in the upper right corner of each graph. Then different purified MAbs (indicated on the abscissa of each graph) were added as inhibitors to the wells in duplicate with ^{125}I-SK. The resulting amount of ^{125}I-SK bound to the immobilized MAb (shown on the ordinate) was determined by gamma-counting.

Figure 2 (A and B): Amino acid sequence of SK and location of peptides. Figure 2, panel A depicts the amino acid sequence of the SK protein (SEQ ID NO: 1). Also indicated by small arrows are the approximate locations of primers used to generate the SK fragments by PCR. The HincII restriction enzyme site is shown. Figure 2, panel B is a schematic illustrating the recombinant SK fragments. The initial and terminal amino acid numbers of each fragment are shown.

Figure 3 (A and B): Electrophoresis and immunoblotting results. Figure 3, panel A, shows the results of the SDS-polyacrylamide (12%, reducing conditions) gel electrophoresis of partially purified MBP-SK fusion proteins, stained with Coomassie Brilliant Blue. Figure 3, panel B, shows the results of an electrophoretic gel which has been immunoblotted with pooled monoclonal antibodies followed by ^{125}I-goat antimouse antibody (panel B). The contents of each lane are shown. Molecular weight markers (kDa) are indicated at left.

Figure 4 (A and B): Effects of MAbs on plasminogen activation by SK. In Figure 4, panel A, MAbs were added prior to formation of SK-plasminogen activator complex. Purified MAbs were premixed with native SK for 20 min. and then added to wells containing plasminogen and a chromogenic substrate (CBS 33.08). The generation of plasmin was monitored by the cleavage of the chromogenic substrate. The percentage residual SK activity was determined as described in the Methods. In Figure 4, panel B, MAbs were added after formation of the SK plasminogen activator

complex. SK and plasminogen were preincubated for 5 minutes and then added to wells of a microtiter plate containing anti-SK MAbs, or a control, inert MAb, plasminogen and chromogenic substrate. The generation of plasmin was monitored and the percent residual activity was determined as described.

Figure 5: Effects of anti-SK MAbs on the binding of SK to plasminogen. Wells of a microtiter plate were coated with purified anti-SK MAbs or no MAb. Various amounts of plasminogen (0 to 1.0 mg/ml), aprotinin (1000 units/ml) and ^{125}I-SK (100,000 cpm) were added to the wells. After 1 hr. of incubation the bells were washed and the amount of bound ^{125}I-SK was determined by gamma counting. The percent inhibition of binding of ^{125}I-SK to the MAbs was computed by reference to wells containing no plasminogen as inhibitor (100% binding) and no MAb (0% binding).

Figure 6: Plasminogen activation by recombinant fragments of streptokinase SK_{1-127} and SK_{14-414}, as well as native SK. Each recombinant fragment or SK was incubated with human plasminogen. Generation of plasmin was monitored by the time-related cleavage of its specific p-nitroanilide substrate, S2251.

Figure 7 (A and B): Binding of electrophoretically separated recombinant MBP-SK proteins to plasminogen. After electrophoresis on 10% SDS-polyacrylamide gels, proteins were stained with Coomassie blue dye (panel A) or electrophoretically transferred to polyvinylidene difluoride membranes and probed with ^{125}I-plasminogen (panel B). The membranes were then subjected to autoradiography to detect bound plasminogen. The lanes contain: lane S, molecular weight standards; lane 1, SK 1-414; lane 2, SK 1-352; lane 3, SK 1-253; lane 4, SK 1-127; lane 5, SK 244-414; lane 6, SK 120-352; and lane 7, MBP. The molecular weights of standards (in kDa) are indicated on the left.

Figure 8: Binding of purified, cleaved recombinant SK fragments to ^{125}I-plasminogen. Wells of a microtiter plate were coated with purified, cleaved SK fragments or control proteins (MBP or bovine serum albumin

(BSA)). After nonspecific binding sites had been blocked with bovine serum albumin, ^{125}I-plasminogen was added to each well. The wells were washed and the bound plasminogen was detected by gamma counting. The data represent the means ± SD of duplicate observations.

5 *Figure 9*: Inhibition of ^{125}I-plasminogen binding to wild-type SK by various purified recombinant SK fragments. Wells of a microtiter plate were coated with wild-type SK. After nonspecific protein binding sites had been blocked, various amounts of recombinant SK fragments, or wild-type SK, were added as inhibitors. Immediately thereafter, ^{125}I-plasminogen was added
10 to the wells. After a 1-hr incubation, the amount of bound ^{125}I-plasminogen was determined by gamma counting. Each point represents the mean of duplicate observations. In this assay 100% plasminogen binding was that occurring in the absence of a recombinant SK inhibitor, and 0% binding was that occurring in the absence of wild-type SK.

15 *Figure 10*: Competitive binding of recombinant SK fragments to plasminogen. Wells of a microtiter plate were coated with SK 244-414. Then the overlapping fragment SK 120-352, or the full-length SK 1-414 (control), was added to the wells as an inhibitor in various concentrations. Subsequently, ^{125}I-plasminogen was added to the wells for 1 h. The wells
20 were washed and the amount of bound plasminogen was determined by gamma counting. Percentage binding was computed as described in Fig. 9.

Figure 11: Binding of plasminogen to SK fragments. Recombinant MBP-SK fragments were immobilized in wells of a microtiter plate. After nonspecific binding sites had been blocked with bovine serum albumin,
25 ^{125}I-plasminogen was added for 1 h. The wells were then washed and gamma counted to detect bound plasminogen. Control wells contained bovine serum albumin only. The data represent the means ± SD of duplicate observations.

Figure 12: Plasminogen activation by various SK fragments. Purified recombinant SK fragments (0-1.0 μg) were mixed with human plasminogen.
30 The generation of plasmin was assayed by continuously monitoring the

cleavage of a specific paranitroanilide substrate of the enzyme. The data represent the means ± SD of duplicate observations.

Definitions

In the description that follows, a number of terms used in biochemistry, recombinant DNA (rDNA) technology and immunology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Gene. A nucleic acid sequence containing a template for a nucleic acid polymerase. The nucleic acid transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA) and, in eukaryotes, is transcribed by RNA polymerase II.

A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of RNA lacking intervening sequences (introns).

Cloning vehicle. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for "cloning vehicle."

Expression vehicle. A vehicle or vector which is similar to a cloning vehicle but is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Expression control sequences will vary depending on whether the

vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

5 Functional Derivative. The present invention pertains to functional derivatives of streptokinase. A "functional derivative" of a SK sequence, either protein or nucleic acid, is a molecule that possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of wild-type SK. The term "functional derivative" is intended to 10 include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, 15 absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art.

20 Fragment. A "fragment" of a molecule such as SK protein or nucleic acid is meant to refer to any portion of a native SK amino acid or nucleotide genetic sequence. Additionally, the term "fragment" includes a chemically synthesized protein fragment of SK.

Variant. A "variant" of a SK protein or nucleic acid is meant to refer 25 to a molecule substantially similar in structure and biological activity to either the native SK molecule, or to a fragment thereof. Thus, provided that two molecules possess a common activity and may substitute for each other, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not 30 identical to that found in the other, or if the amino acid or nucleotide sequence is not identical.

Fusion protein. The term "fusion protein" when applied to the SK fragment of the present invention refers to the product produced from the recombinant gene encoding the SK fragment when it is adjacent to or "fused" to a gene for a carrier protein having no plasminogen activator activity, such that the two genes are "in frame" and yield a single protein, combining the carrier and the SK fragment.

Substantially pure form. The term "substantially pure form" when applied to the SK fragment of the present invention means that the SK fragment is essentially free, i.e., free from any other detectable biological constituents, such as intact host cells with which the SK fragment of the present invention is normally associated.

Immunogenically effective amount. An "immunogenically effective amount" is that amount of SK antigen (SK or a fragment thereof) necessary to induce the production of antibodies which will bind to SK epitopes.

Antigen. The term "antigen" as used in this invention is meant to denote a substance that can induce a detectable immune response to SK when introduced to an animal. Such substances include the SK protein, and fragments thereof.

Epitope. An antibody is said to be capable of binding a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of an antigen which can be recognized and bound by an antibody. An antigen may have one, or more than one epitope. An "antigen" is capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

Antibody. The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable

of binding an antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)).

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Detailed Description of the Invention

A. Construction and Identification of Antibodies to SK

In the following description, reference will be made to various methodologies well-known to those skilled in the art of immunology. Standard reference works setting forth the general principles of immunology include the work of Catty, D. (*Antibodies, A Practical Approach*, Vol. 1, IRL Press, Washington, DC (1988)); Klein, J. (*Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, New York (1982)); Kennett, R., *et al.* in *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, New York (1980)); Campbell, A. ("Monoclonal Antibody Technology," in: *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13 (Burdon, R., *et al.*, eds.), Elsevier, Amsterdam (1984)); and Eisen, H.N., in: *Microbiology*, 3rd Ed. (Davis, B.D., *et al.*, Harper & Row, Philadelphia (1980)).

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Methods for preparing antisera in animals are well-known to those skilled in immunology (see, for example, Chard, *Laboratory Techniques in Biology*, "An Introduction to Radioimmunoassay and Related Techniques," North Holland Publishing Company (1978), pp. 385-396; and *Antibodies, A Practical Handbook*, vols. I and II, D. Catty, ed., IRL Press, Washington, D.C. (1988)). The choice of animal is usually determined by a balance between the facilities available and the likely requirements in terms of volume of the resultant antiserum. A large species such as goat, donkey and horse may be preferred, because of the larger volumes of serum readily obtained. However, it is also possible to use smaller species such as rabbit or guinea pig which often yield higher titer antisera. Usually, subcutaneous injections of the antigenic material (the protein or fragment thereof hapten-carrier protein

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conjugate) are made in order to stimulate the immune system of the animal in which antibodies are to be raised. The detection of appropriate antibodies may be carried out by testing the antisera with appropriately labeled tracer-containing molecules. Fractions that bind tracer-containing molecules are then isolated and further purified if necessary.

Antibodies of the present invention may be prepared by any of a variety of methods. For example, purified SK protein, or a fragment thereof, may be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding to epitopes of the SK protein. If desired, such SK antibodies may be purified from the other polyclonal antibodies by standard protein purification techniques and especially by affinity chromatography with purified SK or fragments thereof.

Monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al., Nature 256*:495 (1975); Kohler *et al., Eur. J. Immunol. 6*:511 (1976); Kohler *et al., Eur. J. Immunol. 6*:292 (1976); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal with SK or a fragment thereof. The splenocytes of immunized animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed; however, it is preferable to employ the parent myeloma cell line (SP_2O), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands, J.R., *et al., Gastroenterology 80*:225-232 (1981). The hybridoma cells obtained are then assayed in order to identify clones which secrete antibodies capable of binding the SK and/or the SK protein antigen. The proliferation of transfected cell lines is potentially more promising than classical myeloma technology, using methods available in the art.

Through application of the above-described methods, additional cell lines capable of producing antibodies which recognize epitopes of the SK protein can be obtained. These antibodies can be used in assays to correlate

SK binding and SK activity with the availability of a particular SK epitope. In this way, information concerning regions of SK which bind to plasminogen may be obtained.

B. Epitope Mapping

Once a series of monoclonal antibodies has been produced, the epitopes of SK can be mapped. Initially, each antibody is tested against each other antibody in a competitive binding assay for SK. Those antibodies which effectively inhibit the binding of each other are grouped together and can be said to be specific for a single epitope of SK. Using this method, one MAb from each epitopic group is selected for testing against the representative MAb of each other group to confirm that each is directed against a distinct epitope of SK. Using the deletion mutants, or recombinant SK fragments, described below, each representative MAb and its corresponding epitope is matched to a region on the SK sequence. While starting with large deletions in the SK sequence, it is within the capability of one skilled in the art to produce progressively smaller and more precise deletions to further narrow the site of each epitope, using the antibody binding assay described herein.

C. Cloning of Streptokinase Fragments Which Bind to Plasminogen

Based upon the binding experiments set forth in Example 2, it was determined that peptides with sequences corresponding to amino acids 1-352; 120-352; 244-414; and 244-352 of streptokinase (Figure 2, SEQ ID NO: 1) bind to plasminogen. The invention is directed, *inter alia*, to these peptides and to the nucleic acids encoding these peptides.

DNAs encoding peptides corresponding to regions of SK which bind to plasminogen may be made synthetically or cloned by any method known in the art (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)). Since both the amino acid and DNA sequences of streptokinase are known, short DNA primers can be synthesized and used

to amplify nucleic acids with the desired sequences from any cell known to contain the streptokinase gene. Procedures for amplifying nucleotide sequences using the polymerase chain reaction (PCR) are well-known in the art (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring
5 Harbor Press (1989)).

A preferred method of cloning the claimed DNAs is set forth in detail in Example 2 and in Example 1, section "c". In this method primers are used to amplify nucleic acid from *Streptococcus equisimilis* using the procedures described by Saiki et al. (*Science 239*:487-491 (1988)) and Reed et al. (*J.*
10 *Immunol. 150:*4407-4415 (1993)). The following pairs of primers were found to be suitable for the amplification and cloning of SK fragments (numbers refer to the amino acid residues; primer restriction endonuclease sites (EcoRV or *Pst*I) are underlined): SK amino terminus (SEQ ID NO: 2), [5'-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxyl terminus (SEQ ID NO: 3),
15 [5'-d(GC CTG CAG TCA TTA TTT GTC GTT AGG)]; SK 244-248 (SEQ ID NO: 4), [5'-d(GC GAT ATC CGT GTT AAA AAT CGG G)]; SK 127-122 (SEQ ID NO: 5), [5'-d(GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; SK 253-248 (SEQ ID NO: 6), [5'-d(GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; SK 352-347 (SEQ ID NO: 7), [5'-d(GC
20 CTG CAG TCA TTA TCC AGT TAA GGT ATA GTC)]; SK 120-124 (SEQ ID NO: 8), [5'-d(GC GAT ATC GAC AAA GAT GGT TCG)]. The amplified DNA may be cloned into a vector in accordance with conventional techniques and sequenced in order to confirm its identity (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

25 D. Expression of Cloned DNAs and Isolation of Peptides

In order to obtain the claimed peptides, the amplified and cloned DNAs can be inserted into an expression vector and the expression vector used to transform an appropriate host cell. A variety of vector/host systems have been described in the literature (Sambrook *et al.*, Molecular Cloning, A Laboratory
30 Manual, Cold Spring Harbor Press (1989)).

Expression vectors typically contain 5' sequences involved in the initiation of transcription and translation. Sequence elements commonly found in expression vectors include promoters, enhancers and upstream activator sequences. Expression vectors may also a encode signal sequence directing 5 the secretion or the compartmentalization of expressed proteins.

The 3'-non-translated region of expression vectors typically contain translation termination sequence elements and, in eukaryotic cells, sequence elements which direct the polyadenylation of mRNA.

The vector containing the nucleic acid encoding the desired SK peptide 10 may be introduced into an appropriate host cell by any of a variety of suitable means, including transfection. After the introduction of the vector, recipient cells are grown in a medium which selects for the growth of vector-containing cells. Expression of the cloned gene sequence results in the production of recombinant protein. This expression can take place in a transient manner, in 15 a continuous manner or in a controlled manner, i.e. expression may be inducible or repressible.

In the preferred embodiment described in Example 2, the cloned DNA is ligated to the pMAL expression vector and expressed as a fusion protein in *Escherichia coli* wherein peptides are fused to a maltose binding protein 20 (MBP; Maina *et al.*, *Gene 74:*365-373 (1988)). The pMAL vector contains the Lac repressor, which allows synthesis to be induced by isopropyl-b-D-thiogalactoside.

Expressed peptides may be purified from the host cell using standard biochemical procedures. Purification is facilitated by the fact that monoclonal 25 antibodies are available which recognize the peptides (see section A above) and because the physical properties of the peptides can be predicted from their known sequence. With respect to the preferred embodiment discussed above, it has been found that SK fusion proteins may be purified by affinity chromatography on an amylose resin, by affinity chromatography on a 30 plasminogen-Sepharose column, or by preparative gel electrophoresis. The

fusion proteins may then be digested with factor Xa and the free peptide isolated.

E. Mutagenesis of SK and SK Peptides

Derivatives of SK or the SK peptides disclosed herein can be prepared
5 by the mutagenesis of DNA. This can be accomplished using one of the mutagenesis procedures known in the art (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

Preparation of derivatives is preferably achieved by site-directed mutagenesis. Site-directed mutagenesis typically employs a phage vector that
10 exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, as disclosed by Messing *et al.*, Third Cleveland Symposium on Macromolecules and Recombinant DNA, A. Walton ed., Elsevier, Amsterdam (1981). These phage are commercially available and their use is well known to those skilled
15 in the art. Alternatively, plasmid vectors containing a single-stranded phage origin of replication (Veira *et al., Meth. Enzymol. 153:*3 (1987)) may be employed to obtain single-stranded DNA.

Site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence the DNA
20 sequence which is to be altered. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al., Proc. Natl. Acad. Sci. (USA) 75:*5765 (1978). The primer is then annealed with the single-stranded vector containing the sequence which is to be altered, and the created vector is incubated with a DNA-
25 polymerizing enzyme such as *E. coli* polymerase I Klenow fragment in an appropriate reaction buffer. The polymerase will complete the synthesis of a mutation-bearing strand. Thus, the second strand will contain the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as JM101 cells, and clones are selected that contain recombinant
30 vectors bearing the mutated sequence.

Whereas the site for introducing a sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at a target region and the newly generated sequences can be
5 screened for the optimal combination of desired activity.

F. Use of the Claimed Nucleic Acids and Peptides

As discussed above, the claimed DNAs can be used to recombinantly produce peptides which have been shown to bind to plasminogen. Using procedures well-known in the art, the DNA molecules can be labeled and used
10 as probes in Northern blots to detect streptokinase mRNA or in Southern blots to detect the presence of the streptokinase gene (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

The peptides may be used in binding assays to detect the presence of plasminogen. Such assays may be performed either using free peptides or
15 peptides which are part of a fusion protein. For example, a cell line may be grown in medium containing radioactively labeled amino acids so that all of the proteins made by the cells are labeled (see Ausubel *et al.*, *Current Protocols in Molecular Biology*, 10.18.1-10.19.1 (1993)). In order to determine if plasminogen is being made, fusion proteins are immobilized by
20 being bound to an appropriate support (e.g. polyvinylidene difluoride membranes, or the wells of a microtiter plate). Nonspecific binding sites on the support are blocked with proteins that do not bind to plasminogen, e.g. by exposing the support to solutions of bovine serum albumin or nonfat milk. The immobilized peptides are then incubated with a cell lysate prepared from
25 the cells grown in the presence of radioactively labeled amino acids. If plasminogen is present in the sample, it will bind to the immobilized peptides and may be detected by washing the support and counting radioactivity.

Alternatively, peptides may be radioactively labeled at tyrosine residues using chloramine T or Iodogen (see Ausubel *et al.*, *Current Protocols in*
30 *Molecular Biology*, 11.16.5-11.16.7 (1993)). Protein samples suspected of

containing plasminogen may be bound to an immobile support such as the wells of a microtiter plate. Nonspecific binding sites may then be blocked as described above and solutions containing the labeled peptides added to the wells. After incubation, the wells may be washed. The amount of plasminogen in the sample would then be determined as the amount of gama radiation remaining in the wells. These types of assays are well-known in the art and are routinely performed by biological laboratories (for a detailed discussion of procedures that can be used, see Ausubel *et al.*, *Current Protocols in Molecular Biology*, 11.2.1-11.2.19 (1993)).

Labeled peptides may also be used to detect the presence of plasminogen *in vivo* by scintigraphy. In this respect, it should be noted that the binding of peptides does not result in the enzymatic activation of plasminogen. Therefore, detection of plasminogen in animals may be accomplished without generating excessive levels of plasmin.

Besides their use in assays designed to detect the presence of plasminogen, the claimed peptides may be used to purify plasminogen. Peptides may be attached to an appropriate chromatography matrix, e.g. Sepharose 4B and then used to purify plasminogen by affinity chromatography. Such procedures are commonly used with monoclonal antibodies and the adaptation of these procedures to use with the claimed peptides is well within the skill of the ordinary biological laboratory (see e.g., Dean *et al.*, *Affinity Chromatography, A Practical Approach*, IRL Press (1986)).

Peptides such as SK_{14-414} which exhibit reduced antigenicity but which maintain the ability to activate plasminogen may be used therapeutically in the same way that intact streptokinase is presently used. Procedures for treating patients with streptokinase are well known in the art (see e.g., Gruppo Italiano per lo Studio della Streptokinase nell'Infarto Miocardico (GISSI), *Lancet* *1*:397-402 (1986); ISIS (Second International Study of Infarct Survival) Collaborative Group, *Lancet* *2*:349-360 (1988); Spottl and Kaiser, *Thromb. Diath. Haemorrh.* *32*:608-616 (1974); and Lynch *et al.*, *Br. Heart J.* *66*:139-142 (1991).

Having now generally described this invention, the same will be further described by reference to certain specific examples which are provided herein purposes of illustration only and are not intended to be limiting unless otherwise specified.

5

Example 1

Methods

Monoclonal Antibody Production

Commercially prepared streptokinase (Hoechst Roussel, Somerville, NJ) was further purified by ammonium sulfate precipitation (40%), followed 10 by ion exchange chromatography on DEAE Affigel Blue (Bio-Rad, Richmond, CA) with a NaCl gradient from 0 to 100 mM. The resulting SK was homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K., *Nature 227*:680-685 (1970)).

Female Balb/C mice (Charles River, Wilmington, MA) were 15 immunized subcutaneously with 33 μg of SK in Freund's adjuvant. They were again immunized 2 weeks later and hyperimmunized 3 weeks later with 3 μg of SK intravenously 2 days prior to fusion. After fusion, cells were distributed into ten 96-well microtiter plates. Hybridoma growth was detected in nearly all wells. Hybridoma supernatants were tested for the presence of 20 anti-SK MAbs in a reverse solid-phase assay (Lukacova *et al.*, *Biochemistry 30*:10164-10170 (1991)). Wells of a microtiter plate were coated with goat antimouse antibody (25 μl, 3 μg/ml). After blocking nonspecific binding sites with 1% BSA, 25 μl of hybridoma supernatant was added for 1 hr. The wells were washed, and ^{125}I-SK (100,000 cpm/25 μl) was added. After 1 hr the 25 wells were again washed and the amount of bound SK was determined by gamma counting. The SK was radioiodinated using the Iodogen method (Pierce, Rockford IL) (Fraker *et al.*, *Biochem. Biophys. Res. Commun. 80*:849-857 (1978)) to a specific activity of $\sim 2 \times 10^6$ cpm/μg. Of the 960 wells tested, 13% produced anti-SK antibody capable of capturing greater than 30 2,000 cpm of ^{125}I-SK (2.5 times control binding). Fifty-one hybridomas

produced Ab that consistently captured > 10,000 cpm (> 10 times control values; apparent $K_d \leq 10^{-8}$ M); these were selected for further study. Hybridomas were cloned by limiting dilution and serotyped using Zymed reagents (San Francisco, CA). The isotype of the categorical MAbs (see below) was $Ig_{\gamma1}K$.

Antibody Binding Assays

a. *Epitope Mapping Assays.* A competitive radioimmunoassay was used to determine whether hybridomas produced Ab that bound to the same epitope on SK. Wells of a microtiter plate were coated with affinity-purified goat antimouse Ab (3 μg/ml) for 90 minutes. Nonspecific protein binding sites were then blocked by incubation with 100 μl of 1% BSA in Tris-buffered saline. The BSA solution was removed and 25 μl of a given hybridoma culture supernatant was added to a series of wells and incubated for 90 minutes. After washing, either culture supernatant from other hybridomas or the same hybridoma (or 1% BSA) was added to these wells as competitors. (The culture supernatant from the same hybridoma was a positive control for inhibition; 1% BSA was a negative control.) Then 25 μl of ^{125}I-SK (~50,000 cpm) was added to the culture supernatants in each well. After 1 hr of incubation the unbound radioactivity was aspirated, the wells were washed, cut and counted. Antibodies were considered to bind to the same epitope if the Ab in the culture supernatant from the second hybridoma inhibited the binding of the ^{125}I-SK to the immobilized Ab (first hybridoma culture supernatant) by more than 50% (typically > 90%). All hybridomas could be classified into categorical or epitopic groups on the basis of these experiments. Based upon this classification, one representative (or categorical) MAb was selected for expansion into ascites. The MAbs were purified by chromatography on protein A-agarose as described (Lukacova *et al.*, *Biochemistry 30*:10164-10170 (1991)). Competition binding studies were repeated using the purified MAbs representing each of different epitopic groups. Plates were coated with 20 μg/ml of purified MAb (25 μl) for one hr. The plates were washed and

nonspecific protein binding sites were blocked with 1% BSA for 30 min. Then purified MAbs (diluted to a final concentration of 10 μg/ml) were mixed with ^{125}I-SK and 25 ml of the mixture (50,000 cpm) was added to the wells containing the immobilized MAb. After 60 minutes of incubation, the reaction mixtures were aspirated. The wells were washed and gamma-counted to determine the amount of bound SK.

b. *Binding of MAbs to SK fragments.* Wells of a microtiter plate were coated with purified SK fragments in a concentration of 20 μg/ml or no antigen. After 90 min. the wells were washed and nonspecific binding sites were blocked with 1% BSA. The wells were washed and incubated with culture media for 1 hr. After washing, bound antibody was detected with ^{125}I-goat antimouse antibody. The competition between human plasminogen and the anti-SK MAbs for binding to ^{125}I-SK was measured in a reverse-solid phase assay. Microtiter plates were coated with 20 μg/ml (25 μl) of purified anti-SK MAbs or no MAb for 2 hrs. in duplicate. After being washed, the plates were blocked with 150 μl of 1% BSA for an hour. Then 25 μl of purified human plasminogen (Deutsch and Mertz, *Science 170*:1095-1096 (1970); with concentrations ranging from 0 to 1.0 mg/ml) in 1% BSA and aprotinin (1000 u/ml) were added to the wells followed immediately by ^{125}I-SK (100,000 cpm/25 μl). After 1 hr of incubation, the wells were washed, cut and counted. The amount of bound ^{125}I-SK was determined by gamma counting. The percent inhibition of binding of ^{125}I-SK to the anti-SK MAb by different concentrations of plasminogen was computed by reference to the amount bound to the MAb in wells containing no plasminogen (defined as 0% inhibition) and no MAb (defined as 100% inhibition).

Production of Recombinant SK Fragments

Bacterial genomic DNA was prepared from *Streptococcus equisimilis*, strain H46A (Lancefield's Group C, ATCC # 12449) and the SK gene was cloned using PCR (Saiki *et al.*, *Science 233*:1076 (1988)). Two synthetic

oligonucleotide primers were designed based upon a previously published SK nucleotide sequence (Malke *et al.*, *Gene 34*:357-362 (1985)). The upstream primer was designed to recapitulate the codons of the N-terminus leader sequence of SK. The downstream primer corresponded to the antisense sequence of the C-terminus of the SK gene and contained two stop codons. A restriction endonuclease site was incorporated at the 5′ end of each primer in order to facilitate cloning. The oligonucleotide primers used for amplification and cloning of the SK gene were: SK leader peptide (SEQ ID NO: 9), [5′-d(CCC AGA TCT ATG AAA AAT TAC TTA TCT TTT GG)]; SK carboxy terminus (SEQ ID NO: 10), [5′-d(CCC GGA TCC TCA TTA TTT GTC GTT AGG GTT ATC AC)]. The cloned SK DNA was sequenced (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA 74*:5463-5467 (1977)) and found to be identical to the published sequence.

Truncated portions of the SK gene were generated in a similar manner using PCR and specific internal primers. The following primers were used for amplification and cloning of SK fragments (numbers refer to the amino acid residues): SK amino terminus (SEQ ID NO: 2), [5′-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxy terminus (SEQ ID NO: 3), [5′-d (GC CTG CAG TCA TTA TTT GTC GTT AGG)]; $SK_{244-248}$(SEQ ID NO: 4), [5′-d (GC GAT ATC CGT GTT AAA AAT CGG G)]; $SK_{127-122}$ (SEQ ID NO: 5), [5′-d (GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; $SK_{253-248}$ (SEQ ID NO: 6), [5′-d (GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; $SK_{352-347}$ (SEQ ID NO: 7), [5′-d (GC CTG CAG TCA TTA TCC ACT TAA GGT ATA GTC)]. The primer restriction endonuclease sites are underlined (EcoRV or Pst 1). An internal Hinc II site in the SK gene was also used in order to generate a protein fragment which began with a valine residue, the amino acid in position 14 of full-length SK. After the DNA sequence corresponding to full length SK or the desired SK fragment was cloned, it was ligated into the pMAL expression vector (New England Biolabs, Beverly, MA) and expressed in *E. coli* as a fusion protein (with maltose binding protein, MBP; Maina *et al.*, *Gene 74(2)*:365-73 (1988)). This vector

also contained the Lac repressor, allowing induced synthesis by isopropyl-β-D-thiogalactoside.

The SK fusion proteins were purified by affinity chromatography on an amylose resin as described by the supplier, or by chromatography on a
5 DEAE Affigel Blue agarose (BioRad, Hayward, CA) and the purity was assessed by SDS-PACE (Laemmli, U.K., *Nature 227*:680-685 (1970)). Following SDS-PAGE, SK proteins were stained with Coomassie blue dye or transferred to polyvinylidene difluoride membranes by semi-dry electroblotting as described (Khyse-Anderson, J., *J. Biochem. Biophys Meth. 10*:203-209
10 (1984)). The membranes were blocked in 5% nonfat milk and probed with pooled anti-SK hybridomas supernatants for 1 hr. After washing 6 times, the membranes were incubated in ^{125}I-goat anti-mouse antibody (1,000,000 cpm) for 1 hr. After additional washing, the membranes were exposed to Kodak X-Omat film (Rochester, NY) at -70^0 C.

15 Effects of MAbs on SK Activity

Purified MAb (25 μl, 1 mg/ml) was mixed with native SK (25 μl, 1 mg/ml) for 20 minutes at room temperature and then added to wells of a microtiter plate containing 25 μl each of 0.1% BSA in Tris-buffered saline, human plasminogen (100 μg/ml) and CBS 33.08 substrate (AcOH.H-D-
20 NLeuCHA-Arg-pNA, 1.9 mM, Diagnostica Stago, Asnieres-Sur-Seine, France). The rate of plasminogen activation to plasmin was determined by the cleavage of the plasmin substrate CBS 33.08 and subsequent release of the p-nitroanilide product. The rate of p-nitroanilide production was recorded continuously at 405 nm in triplicate for each of these samples in a microtiter
25 plate reader (Thermomax, Molecular Devices, Palo Alto, CA). The rate of p-nitroanilide formation in the presence of a given MAb was compared to the rate of product formation in the presence of a purified, inactive, control antidigoxin MAb (100% activity) or in absence of any SK (0% activity). In other experiments, the effect of the MAbs was studied on the preformed SK-
30 PAC. SK (25 μl, 1 μg/ml) was mixed with human plasminogen (25 μl, 2

μg/ml) for 5 min. and added to wells containing 25 μl each of MAb (1 mg/ml), human plasminogen (1 mg/ml) and CBS substrate. The rate of plasminogen activation was measured as described above.

Streptokinase Activity of Recombinant SK Fragments

5 Recombinant SK fragments SK_{1-127} and SK_{14-414}, as well as native SK, were incubated with human plasminogen. The generation of plasmin was monitored by the time-related cleavage of its specific p-nitroanilide substrate, S2251.

Results

10 From the fused splenocytes of one immunized mouse, 121 hybridomas were found to produce antibody that specifically bound to SK at levels greater than 2.5 times background in a reverse-solid phase radioimmunoassay. Of these, 51 hybridomas consistently 'captured' ^{125}I-SK at levels exceeding ten times control. The epitope binding specificities of these hybridomas were 15 determined by competitive binding experiments. In this assay, an Ab from one hybridoma was immobilized in the wells of a microtiter plate. Then culture supernatant from another hybridoma was added to compete with the immobilized Ab for binding to ^{125}I-SK. Virgin culture media and culture supernatants from the same hybridoma were used as negative and positive 20 controls for inhibition, respectively. Antibodies were considered to bind to the same epitope if the Ab in the culture supernatant inhibited the binding of the ^{125}I-SK to the immobilized Ab by more than 50%. (Typically the inhibition was 90%). These competitive inhibition assays revealed that the hybridomas could be classified into six major epitopic groups. From each group one 25 representative or categorical MAb was selected for further study. These MAbs were expanded into ascites and purified using affinity chromatography. Competitive binding assays were performed with each of these purified MAbs to verify that they bound to different epitopes. Purified MAb was immobilized in wells of a microtiter plates. After blocking nonspecific binding, each of the

purified MAbs was added in duplicate to the wells with ^{125}I-SK. Figure 1 shows that the binding of the immobilized MAb to ^{125}I-SK was inhibited when the same MAb was used as competitor (self-inhibition). But when other MAbs were used as competitor, they caused little or no effect. Thus, these six categorical MAbs appeared to bind to sterically distinct or minimally overlapping epitopes.

To locate the peptide sequences composing the epitopes recognized by these MAbs, SK fragments were generated using PCR and expressed in *E. coli*. The amino acid sequence of native SK is shown in Figure 2A. Above and below the amino acid sequence is the approximate position of the sense and antisense primers used to amplify the DNA coding for the SK fragments. Figure 2B displays the expressed recombinant SK fragments in schematic form. The NH_2-terminal and COOH-terminal amino acid residues of the SK fragments are indicated by number. Figure 3A shows Coomassie blue-stained gels demonstrating the expression of these induced fusion proteins containing the SK fragments. Figure 3B shows the same material subjected to immunoblotting with pooled MAbs against SK. These two figures demonstrate that the major protein product in the induced *E. coli* lysates is the SK fusion protein. All of the SK fusion proteins are of appropriate molecular mass and are immunoreactive with the SK MAbs, while the other component of the fusion protein, MBP, is not reactive.

The binding patterns of the categorical MAbs to these SK fragments were studied in a direct binding assay. The results are tabulated in Table I.

TABLE I. BINDING OF SK MABS TO SK FRAGMENTS

	Monoclonal Antibody*					
SK fragment	7D4	8F5	8G3	9D10	9H10	10E1
SK_{1-127}	-	-	-	+	-	+
SK_{1-253}	+	-	-	+	-	+
SK_{1-352}	+	+	-	+	+	+
SK_{1-414}	+	+	+	+	+	+
SK_{14-414}	+	+	+	-	+	+
$SK_{244-414}$	-	-	+	-	-	-
Epitope**	128-253	120-352	353-414	1-13	120-352	14-127
No. Abs***	6	10	3	5	12	15

*A '+' indicates that the MAb showed significant binding to the SK fragment (typically 10 times background) in a radioimmunoassay. A '-' sign indicates that the MAb did not bind to the SK fragment at levels significantly greater than background.

**The epitope recognized by the antibody is inferred from the pattern of binding indicated in the table, or by additional data presented in Results.

***The total number of antibodies that bind to the same epitope is listed.

MAb 9D10 binds to full length SK (SK_{1-414}) but does not bind to SK_{14-414}, which lacks the first 13 amino terminal amino acids. This would suggest that MAb 9D10 recognizes an epitope largely constructed by the first 13 amino acids of full-length SK. MAb 8G3 binds to $SK_{244-414}$, but not to SK_{1-352}, suggesting that it recognizes an epitope constructed by amino acid residues in the carboxy terminal end of SK within amino acids 353 to 414. Since MAb 10E1 binds to SK_{1-127}, as well as to SK lacking the first 13 amino acids, it appears to recognize an epitope formed by amino acids 14-127. Two MAbs, 8F5 and 9H10, although they bound to different epitopes by

competition assays, had a similar pattern of binding to the SK fragments, as did all of the MAbs in their respective epitopic groups. Neither 8F5 nor 9H10 bound to the overlapping fragments of SK_{1-253} or $SK_{244-414}$, but both MAbs bound to SK_{1-352}. Later experiments (not shown) also revealed that they bound to another SK fragment, $SK_{120-352}$. Thus it would appear that they recognize an epitope constructed by the association of amino acid sequences on both sides of 244-253 region. Finally, MAb 7D4 appears from its pattern of binding to recognize an epitope constructed by amino acids 128-243. Similar direct binding studies were performed with the other 45 MAbs raised against SK. These MAbs bound to SK fragments in a pattern consistent with the epitope group assignment determined by the competitive binding assays described above (data not shown).

Assays were performed to determine the effects of the MAbs on the formation of the SK-PAC and on the rate of plasminogen activation once the activator complex was formed. When purified MAbs were incubated with SK prior to the addition of plasminogen, all but one MAb appeared to inhibit the rate of plasmin generation (Figure 4A). MAbs 7D4 and 8F5 inhibited plasminogen activation by >90%. MAbs 8G3 and 9D10 also inhibited plasminogen activation by 60-75%, while MAb 10E1 had minor effects. Only MAb 9H10 had no apparent effect on SK activity. However, when the MAbs were added after the SK-PAC was allowed to form, only one of the MAbs, 8F5, showed significant inhibition of plasminogen activation (Figure 4B). This suggested that MAb 8F5 interfered with the catalytic function of the SK-PAC, while the other MAbs inhibited SK activity by interfering with the binding of SK to plasminogen during the formation of the SK-PAC. To test this hypothesis, the binding of ^{125}I-SK to plasminogen was measured in the presence and absence of the monoclonal antibodies (Figure 5). This graph shows that MAbs 7D4 and 8F5 were effective inhibitors of the binding of ^{125}I-SK to human plasminogen at plasminogen concentrations up to, and slightly in excess of, the concentration of plasminogen in plasma (210 mg/ml). The

other MAbs showed no significant competition with SK-plasminogen binding at these concentrations.

Figure 6 shows the plasminogen activation by recombinant SK fragments SK_{1-127} and SK_{14-414}, as well as native SK. SK_{1-127} showed no apparent plasminogen activating ability, compared to the control, whereas SK_{14-414} shows preserved plasminogen activating capacity.

Discussion

Streptokinase is now widely used as an effective treatment for dissolving thrombi that cause heart attacks. Although its immunogenicity has been evident since the 1930s, the molecular basis for its antigenicity has not been studied. The present invention provides a panel of 51 murine hybridomas producing Abs against the native protein as probes to begin to analyze the humoral immune response to SK. These 51 hybridomas were initially selected at somatic cell fusion because of their avid binding to ^{125}I-SK in a reverse solid phase assay. Because this type of assay avoids solid-phase immobilization of SK, it should allow all solution-phase epitopes of the protein to be available for binding to the MAbs. Competition binding assays (performed in the same fashion) demonstrated that these MAbs could be grouped into 6 major complementation or epitope groups that are spatially or sterically distinct. The assignment of these MAbs to epitope groups by competition binding studies was confirmed by their characteristic pattern of binding to SK fragments generated by recombinant DNA techniques. The epitopes recognized by most of the categorical MAbs could be localized to a specific SK fragment, as noted in Figure 1. Correlation of the epitope mapping studies with the functional effects of the MAbs on SK activity suggested regions of the SK molecule which may play a critical role in plasminogen binding and the catalytic function-of the SK-PAC.

The anti-SK MAbs studied are by nature a 'selected' sample of the secondary, polyclonal antibody response to SK in these inbred mice. By studying a large number of hybridomas, we sought to derive MAbs which

would be representative of the polyclonal antibody response. In turn, the epitopes identified by these MAbs should be broadly indicative of the major epitopes recognized by the polyclonal antibody response. Previous studies have demonstrated that MAbs can be used advantageously to identify peptide regions important for a molecule's immunogenicity and function (Benjamin *et al.*, *Annu. Rev. Immunol.* *2*:67-101 (1984)). Data from these studies provide important functional information which complements the insights gained from studies of the protein's three-dimensional structure. In this study, the epitope pattern of 51 hybridomas was first determined in a competitive liquid phase assay and then confirmed by the pattern of binding of the hybridomas to recombinant fragments of SK. Using these techniques, each of the Abs studied could be assigned to one major epitopic group with little or no overlap between groups (see Figure 1 and Table I). Considering the relative number of MAbs directed to epitopes throughout the molecule (Table I), it would appear that the amino terminal sequence of SK constructs epitopes that may be 'more antigenic' than the epitopes constructed by the carboxy terminal sequence. For example, considering its relatively small size, the amino terminal 13 amino acids of SK appeared to construct, or provide the critical residues for, an epitope recognized by a surprisingly large number of MAbs. The small size of this region suggests that this may be a 'continuous' epitope dependent on the actual linear sequence of amino acid residues in this peptide, or that these residues provide most of the binding energy responsible during contact with the MAbs. Others have also noted that about 10% of the antibodies raised against a protein antigen will bind to a peptide fragment of the protein (Pellequer *et al.*, *Methods Enzymol.* *203*:176-201 (1991)). Deletion of these 13 residues appeared to cause only a local change in SK structure since all the other categorical Abs continued to bind to the SK_{14-414} fragment.

An explanation for the antigenicity of the amino terminus may be related to its structure. Although the actual three-dimensional structure of SK is not known, it is likely to be similar to the crystal structure of *Streptomyces*

griseus protease A, as suggested by Jackson and Tang, *Biochemistry 21(26)*:6620-5 (1982)). In the crystal structure of *Streptomyces griseus* protease A, the structure of the first 13 amino acids consists of a two reverse turns connected by a β-strand. This segment is among the most solvent accessible regions in the *Streptomyces griseus* protease A molecule. In addition, there are three charged residues in this 13 amino acid segment of SK (Figure 2A) and one aromatic residue (the only tryptophan in the SK molecule). These residues may be important factors in the antigenicity of this region since it has been shown that both charged residues and aromatic residues play a significant role in binding through salt bridges and hydrophobic interactions (Sheriff *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8075-9 (1987); Amit *et al.*, *Science 233*:747-53(1986)).

The other epitopes in the molecule appear to be more discontinuous in nature. The best examples are the epitopes recognized by 8F5 and 9H10. Neither of these MAbs bound to overlapping fragments representing SK_{1-253} or $SK_{244-414}$. Rather they appeared to recognize an epitope constructed by the interaction of amino acids in the peptide sequences on both sides of the region of 244-253. Finally, though all 51 MAbs could be grouped into 6 major epitopic groups, it is likely that many of these MAbs display differences in their fine binding specificity which could be demonstrated by more subtle changes in the amino acid sequence of SK.

As an additional means of probing the antigenic epitopes of SK we studied the effects of these MAbs on the binding of native SK to plasminogen and on the catalytic activity of SK-PAC. By correlating the MAb binding site data with functional effects of the MAbs we may begin to formulate some hypotheses about the functional regions of SK. For example, MAb 7D4 is a strong inhibitor of the binding of SK to plasminogen but does not appear to affect the catalytic activity of the SK-PAC once it is formed. This suggests that the region recognized by MAb 7D4, amino acids 128-243, may contain a plasminogen binding site. Similarly, MAb 8F5 inhibits the binding of plasminogen to SK as well as inhibiting the catalytic activity of the SK-PAC.

This suggests that amino acids critical to the function of the SK-PAC reside in the region of amino acids 120-352. Further studies using SK fragments can build on these findings to more precisely delineate the structural regions responsible for SK function.

5 Although there is a growing clinical literature on allergic reactions to SK, this report represents the first attempt to dissect the molecular basis of its antigenicity. SK is unique among previously studied model antigens because humans are naturally immunized with it as a result of streptococcal infections. More recently, immunization has occurred as a consequence of the use of SK 10 as a treatment for thrombosis. This natural immunization process facilitates the analysis of the immune response to SK in humans as wells as other mammals.

SK also has other advantages as a model antigen. Its complete amino acid and DNA sequences are known (Malke *et al.*, *Gene 34*:357-362 (1985); 15 Jackson and Tang, *Biochemistry 21(26)*:6620-5 (1982)). It has no carbohydrate chains nor cysteine amino acids; thus recombinant SK produced in bacteria should be antigenically similar to the native molecule. Further, the several naturally occurring variations in SK sequence from different streptococcal strains may allow additional insights into the antigenicity and 20 function of the molecule. Finally, lessons learned from the study of SK in the laboratory may be tested and applied to humans treated with SK as well as those suffering from streptococcal disease.

Example 2

Experimental Procedures

25 Cloning and Expression of SK and SK Peptides

The SK gene was cloned from *Streptococcus equisimilis* by the polymerase chain reaction (PCR) (Saiki et al., *Science 239*:487-491 (1988) as previously described (see Example 1, sections c-e). The cloned SK gene was sequenced (U.S. Biochemicals, Cleveland, Ohio; Sanger *et al.*, *Proc. Natl.*

Acad. Sci. U.S.A. 74:5463-5497 (1977)) and found to be identical to the published sequence (Malke et al., *Gene 34*:357-362 (1985)).

Truncated portions of the SK gene were generated in a similar manner using PCR and specific internal primers. The following primers (Genosys, The Woodlands, TX) were used for amplification and cloning of SK fragments (numbers refer to the amino acid residues; primer restriction endonuclease sites (*Eco*RV or *Pst*I) are underlined): SK amino terminus (SEQ ID NO: 2), [5'-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxyl terminus (SEQ ID NO: 3), [5'-d(GC CTG CAG TCA TTA TTT GTC GTT AGG)]; SK 244-248 (SEQ ID NO: 4), [5'-d(GC GAT ATC CGT GTT AAA AAT CGG G)]; SK 127-122 (SEQ ID NO: 5), [5'-d(GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; SK 253-248 (SEQ ID NO: 6), [5'-d(GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; SK 352-347 (SEQ ID NO: 7), [5'-d(GC CTG CAG TCA TTA TCC AGT TAA GGT ATA GTC)]; SK 120-124 (SEQ ID NO: 8), [5'-d(GC GAT ATC GAC AAA GAT GGT TCG)].

After the DNA sequence corresponding to full-length SK, or to the desired SK fragment, had been cloned, it was ligated into the pMAL expression vector (New England Biolabs, Beverly, MA) and expressed as a fusion protein in *Escherichia coli* (with maltose binding protein (MBP; Maina et al., *Gene 74,* 365-373 (1988)). This vector also contained the Lac repressor, which allows induced synthesis by isopropyl-b-D-thiogalactoside.

SK fusion proteins were purified by affinity chromatography on an amylose resin (New England Biolabs) as described by the supplier, by affinity chromatography on a plasminogen-Sepharose column, or by preparative gel electrophoresis on a BioRad Model 491 Prep Cell (BioRad, Richmond, CA). The purity of the recombinant SK fusion proteins was assessed by SDS-PAGE (Laemmli, *Nature 227*:680-685 (1970)).

The SK fusion proteins were cut with factor Xa as described (Maina et al., *Gene 74*:365-373 (1988)). After purification, the amount of recombinant SK 1-414 was determined using an absorption coefficient of 7.5

for a 1% solution at 280 nm (Reddy, *Enzyme 40,* 79-89 (1988)). The relative concentrations of the cleaved, purified SK fragments were determined by comparative radioimmunoassay with fragment-specific monoclonal antibodies (see Example 1). Briefly, wells of a microtiter plate were coated with various concentrations of SK 1-414 (0, 2.5, 5, 10, 20, and 40 μg/mL). After nonspecific binding sites had been blocked with 1% bovine serum albumin, fragment-specific monoclonal antibodies were added to each well in duplicate. After a 1 h incubation the wells were washed and probed with ^{125}I goat anti-mouse antibody (Cappel Organon Teknika, Durham, NC) for 1 h. After another wash the amount of bound antibody was determined by gamma counting. A standard curve relating antibody binding to SK concentration was derived for each fragment-specific monoclonal antibody. The assay was then repeated with unknown concentrations of purified recombinant SK fragments and appropriate fragment-specific monoclonal antibodies. The concentration of a recombinant SK fragment was determined by reference to the standard curve.

Protein Labeling

Human plasminogen (Sigma, St. Louis, MO) was labeled by the Iodogen method (Pierce, Rockford, IL; Fraker and Speck, *Biochem. Biophys. Res. Commun. 80*:849-857 (1978)) to a specific activity of 5500 cpm per ng. Goat anti-mouse antibody was similarly labeled, with specific activities ranging from about 7000 to 10,000 cpm per ng.

Immunoblotting and Ligand Blotting

Purified SK fusion proteins were subjected to electrophoresis on 10% polyacrylamide gels (Laemmli, *Nature 227*:680-685 (1970)). Proteins were stained with Coomassie blue dye or transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semi-dry electroblotting as described (Kyhse, *J. Biochem. Biophys. Meth. 10*:203-209 (1984)). The membranes were blocked in 5% nonfat milk and probed with ^{125}I-plasminogen (2,000,000

cpm) for 1 h. After the blots had been washed they were subjected to autoradiography.

Plasminogen Binding Assays

The binding to plasminogen of purified recombinant SK fragments and
5 MBP-SK fusion proteins was determined as follows. Wells of a microtiter plate were coated with 25 μL of purified recombinant SK fragment at a concentration of 10 μg/mL for 3 h at room temperature. Control wells were coated with purified MBP (0.5 μg/mL) or no antigen. The wells were washed and nonspecific protein binding sites were blocked with 200 μL of 1% bovine
10 serum albumin for 1 h. The wells were washed again and ^{125}I-plasminogen (1-200,000 cpm) was added to each well for 1 h. The unbound plasminogen was removed, the wells were washed, and the amount of bound plasminogen was determined by gamma counting.

The competitive binding to plasminogen of various recombinant SK
15 fragments was also studied. Wells of a microtiter plate were coated with wild-type SK (25 μL, 1 μg/mL) for 1 h. Nonspecific protein binding sites were blocked by adding 200 μL of 1% bovine serum albumin for 1 h. Then 25 μL of various concentrations of recombinant SK fragment (ranging from 0 to 0.835 mg/ml) or no SK (all diluted in 1% BSA in Tris-buffered saline) was
20 added to wells in duplicate. Immediately thereafter 25 μL of ^{125}I-plasminogen (50,000 cpm) was added to each well. After 1 h of incubation the wells were washed and bound ^{125}I-plasminogen was counted in a gamma counter. Wells coated with 1% bovine serum albumin (Sigma, St. Louis, MO) and no SK were used as negative controls. The percentage of plasminogen binding was
25 determined by computing the fractional binding of ^{125}I-plasminogen to SK in the presence of a given inhibitor in comparison with that occurring in the absence of an inhibitor, after correcting for nonspecific binding.

In another assay we studied the ability of SK 120-352 to inhibit the binding of SK 244-414 to plasminogen. Wells of a microtiter plate were
30 coated in duplicate with 20 μg/ml of SK 244-414 for 2 h. Nonspecific protein

binding sites were blocked with 1% bovine serum albumin (200 μL) for 1 h. Then SK 120-352 (25 μL, various concentrations) or no inhibitor was added to the wells in duplicate. Immediately thereafter ^{125}I-plasminogen (25 μL, 50,000 cpm) was added to the wells. After 1 h of incubation, the wells were washed and the amount of bound plasminogen was measured in a gamma counter. The percentage of plasminogen binding was determined as described above.

Plasminogen Activation

Purified, cleaved SK fragments (50 μL, 0 to 5 μg) or no SK was added to wells of a microtiter plate in duplicate. Next 50 μL of plasmin substrate 2-AcOH.H-D-Nleu-CHA-Arg-pNA (1.9 mM; Diagnostica Stago, Asnières-sur-Seine, France) and 50 μL of plasminogen (1 mg/ml; Deutsch & Mertz, 1970) was added to the wells. For each of these samples, the rate of paranitroanilide production was recorded continuously at 405 nm (in duplicate) in a microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA).

RESULTS

SK fragments were generated by PCR (Saiki et al., *Science 239*:487-491 (1988)) and expressed as fusion polypeptides with MBP (at the amino terminus). Fig. 7A shows the purified MBP-SK proteins on a Coomassie blue-stained gel. The expected molecular masses for the purified MBP-SK proteins were: MBP-SK 1-414, 99 kDa; MBP-SK 1-352, 82 kDa; MBP-SK 1-253, 70 kDa; MBP-SK 1-127, 56 kDa; MBP-SK 120-352, 69 kDa; MBP-SK 244-414, 62 kDa; and MBP-LacZ (no SK), 53 kDa. All the expressed proteins were of the expected molecular masses, except for the MBP-SK 1-414 protein, which consistently migrated slightly farther than expected.

In order to determine which MBP-SK fragments bound to human plasminogen, ligand blotting studies were performed. MBP-SK fragments were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then probed with

^{125}I-human plasminogen. Fig. 7B shows that full-length SK (SK 1-414) bound plasminogen, as did the fragments SK 1-352, SK 244-414, and SK 120-352. Fragments SK 1-127 and SK 1-253, and MBP alone did not show significant binding to plasminogen.

5 In order to determine whether the binding of MBP-SK fragments SK 1-127 or SK 1-253 was affected by the presence of the MBP moiety, the fusion proteins were cleaved with factor Xa. After purification, the binding of the cleaved SK fragments (i.e., without MBP) was studied in a solid-phase radioligand assay. Fig. 8 shows that, compared with the negative control 10 proteins (bovine serum albumin and MBP), SK fragments 1-414, 1-352, 120-352, and 244-414 all showed significant binding to plasminogen, but SK 1-127 and 1-253 did not. These results confirmed the previous ligand blotting results with the MBP-SK fusion proteins and argued that the MBP moiety did not prevent binding of the SK fragments to plasminogen.

15 A series of inhibition experiments with purified, cleaved SK fragments was performed to determine the location of plasminogen binding sites. In this series, the ability of SK fragments to interfere with the binding of wild-type SK to ^{125}I-plasminogen was examined. Fig. 9 shows that SK 1-414 fully inhibited the binding of wild-type SK. In addition, recombinant SK fragments 20 1-352, 120-352, and 244-414 caused complete (i.e., $\geq$ 90%) inhibition of plasminogen binding to wild-type SK. However, recombinant SK fragments 1-127 and 1-253 did not show significant inhibition at these concentrations. Because recombinant SK fragments 1-352, 120-352, and 244-414 completely inhibited the binding of plasminogen to wild-type SK, it was inferred that each 25 of the three fragments contained the same plasminogen binding site(s) as native SK.

The smallest fragment that effectively bound plasminogen in these experiments was SK 244-414. Since the next smallest fragment that also bound plasminogen was SK 120-352, it was postulated that the plasminogen 30 binding site(s) were in a region of SK shared by these two fragments, i.e. SK 244-352. To test this hypothesis the ability of SK 120-352 to completely

inhibit the binding of plasminogen to SK 244-414 was examined. Fig. 10 shows that SK 120-352, like full-length SK 1-414, completely inhibited the binding of plasminogen to SK 244-414. This suggested that the region shared by these two fragments, SK 244-352, contained most of the residues necessary
5 for plasminogen binding in these assays.

To investigate this possibility, the SK 244-352 fragment was expressed using the same vector. Fig. 11 compares the binding of SK 244-352, SK 120-352, or no antigen to ^{125}I-plasminogen in a radioimmunoassay. In comparison with control wells, wells containing SK 244-352 and SK 120-352
10 showed significant binding to plasminogen, confirming that the smaller fragment contained the plasminogen binding site(s).

Enzymatic assays were performed to determine whether binding of an SK fragment to plasminogen was sufficient to generate a functional plasminogen activator complex (Figure 12). The original cleaved SK
15 fragments were incubated with human plasminogen and the generation of plasmin was monitored by recording the rate of cleavage of a chromogenic substrate. In this assay, SK 1-414 showed a dose-dependent activation of plasminogen. However, the fragments that bound to plasminogen, SK 1-352, SK 244-414, and SK 120-352, showed no discernible ability to activate
20 plasminogen.

DISCUSSION

Despite its long and extensive use as a therapeutic agent, the molecular mechanisms by which SK acts to form a plasminogen activator remain unknown. Unlike tPA and urokinase, two other widely used plasminogen
25 activators that are serine proteases, SK forms a tight, noncovalent complex with plasminogen to construct a functional plasminogen activator ($K_d = 5 \times 10^{-10}$ M; Wiman, *Thromb. Res. 17*:143-152 (1980)). In Example 1, it was reported that monoclonal antibodies that bind to the regions of SK spanning amino acid residues 120 to 352 inhibited the binding of SK to plasminogen
30 (see also, Reed et al., *J. Immunol. 150:*4407-4415 (1993)). This observation

suggested that the 120-352 region of SK could be involved in plasminogen binding.

In the present experiments, binding studies with SK deletion mutants showed that SK 1-352, SK 120-352, and SK 244-414 also bound to plasminogen and fully inhibited the binding of wild-type SK to plasminogen. This implied that these three fragments contained all the moderate- to high-affinity plasminogen binding sites. Mutual inhibition studies showed that the smallest of the overlapping fragments, SK 120-352 and SK 244-414, competed with each other for binding to plasminogen. Thus, the region of SK shared by the two fragments, SK 244-352, appeared to contain the plasminogen binding site(s). To confirm this observation, SK 244-352 was expressed and found to bind to plasminogen.

The experiments herein suggest that the SK 244-352 region is the primary moderate- to high-affinity binding region in SK for plasminogen. The assays used could have detected a binding interaction in which the binding affinity of the SK fragments for plasminogen was greater than about 10^5 M^{-1} (Eshar, in *Hybridoma Technology in the Biosciences and Medicine* (Springer, T. A., Ed.) pp 3-41, Plenum, New York (1985)). No significant direct binding of plasminogen to the regions of SK spanned by the fragments SK 1-127 or 1-253 was detected. In addition, recombinant SK fragments sharing the common region 244-352 were able to completely inhibit the binding of wild-type SK to plasminogen, arguing that another section of SK did not contribute significantly to plasminogen binding.

Nevertheless, there were notable differences in the avidity with which recombinant SK fragments 1-352, 120-352, and 244-414 bound to plasminogen (Fig. 8). These differences suggest that there may be a second binding site in SK which was induced by the initial binding of the 244-352 region to plasminogen. Alternatively, amino acid sequences outside the 244-352 region may be necessary for obtaining an SK conformation optimal for plasminogen binding. Because deletion mutants will not necessarily assume the optimal conformation for binding to plasminogen, no attempt was made to precisely

measure their individual affinities for plasminogen. Rather, by determining which fragments bound plasminogen, a region of SK was identified that may be more intensively explored in the future by site-directed mutagenesis.

5 There have been surprisingly few studies correlating the structure and function of SK. A few studies have examined the activity of SK proteolytic fragments. For example, Klessen et al. (*Mol. Gen. Genet. 212*:295-300 (1988)) showed that deletion of the first 15 amino acid residues in SK did not affect its activity. Similarly, Brockway and Castellino noted that SK lacking the first 59 amino acids was still a functional plasminogen activator

10 (*Biochemistry 13*:2063-2070 (1974)). Jackson et al. (*Biochemistry 25*:108-114 (1986)) demonstrated that deletion of the carboxyl terminal amino acids (beyond residue 383) did not abolish function. Jackson and Tang noted that there was amino acid sequence homology between the amino terminal and carboxyl terminal halves of SK (*Biochemistry 21*:6620-6625 (1982)). They

15 suggested that SK may have two similar domains, each of which binds plasminogen (Jackson et al., in *The Regulation of Coagulation* (Mann, K. G., & Taylor, F. B., Jr, eds.) pp 515-520, Elsevier, North Holland (1980)), and that these domains may have arisen by gene duplication (Jackson and Tang, *Biochemistry 21*:6620-6625 (1982)).

20 The results disclosed herein do not support this model, since the amino terminal half of SK, as represented by SK 1-253, did not bind to plasminogen. Also, the hypothesis of gene duplication has not been supported by analysis of the SK DNA sequence (Malke et al., *Gene 34*:357-362 (1985)). The present results indicate that the mere binding of plasminogen to SK is not sufficient for

25 the construction of a functional SK-plasminogen activator complex. Unless plasminogen binds to some other occult, low-affinity, or inducible binding site in SK, it appears unlikely that SK merely functions as an allosteric activator or effector.

A more likely hypothesis is that SK binds to plasminogen and

30 contributes amino acids to the active site of the plasminogen activator complex. This possibility was first suggested by Jackson and Tang

(*Biochemistry 21*:6620-6625 (1982)), who noted that SK had some homology with serine proteases. By comparing the amino acid sequence of SK with those of other serine proteases, Jackson and Tang theorized that the usual active site residue histidine was replaced by a glycine at position 24 in SK.

5 To investigate this hypothesis, Lee et al. mutated glycine-24 to a histidine, a glutamine, or an alanine residue (*Biochem. Biophys. Res. Commun. 165*:1085-1090 (1989)). Rather than restore the "lost" serine protease activity of SK, the glycine-to-histidine and glycine-to-glutamine mutations caused almost complete loss of activity, but had no effect on
10 plasminogen binding. Interestingly, the glycine-to-alanine mutation had no effect on activity or plasminogen binding. Although these observations about site-specific mutations do not support assertions that SK has evolved from a serine protease, they do indicate that minor mutations, which do not affect plasminogen binding, have profound effects on the function of SK as a
15 plasminogen activator. Therefore, these observations support the present finding that the mere binding of SK to plasminogen is insufficient for formation of a functional SK-plasminogen activator complex.

All references cited herein are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the
20 art that the scope may be performed within a wide and equivalent range of conditions, parameters and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Reed, Guy L.

(ii) TITLE OF INVENTION: Peptides Specifically Binding to Plasminogen And the DNA Encoding Such Peptides

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Sterne, Kessler, Goldstein and Fox
(B) STREET: 1100 New York Avenue, N.W.
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(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: (to be assigned)
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sanzo, Michael A.
(B) REGISTRATION NUMBER: 36,912
(C) REFERENCE/DOCKET NUMBER: 0609.3570001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 371-2600
(B) TELEFAX: (202) 371-2540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 414 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ile Ala Gly Pro Glu Trp Leu Leu Asp Arg Pro Ser Val Asn Asn Ser
1               5                   10                  15

Gln Leu Val Val Ser Val Ala Gly Thr Val Glu Gly Thr Asn Gln Asp
            20                  25                  30

Ile Ser Leu Lys Phe Phe Glu Ile Asp Leu Thr Ser Arg Pro Ala His
        35                  40                  45

Gly Gly Lys Thr Glu Gln Gly Leu Ser Pro Lys Ser Lys Pro Phe Ala
    50                  55                  60

Thr Asp Ser Gly Ala Met Ser His Lys Leu Glu Lys Ala Asp Leu Leu
65                  70                  75                  80

Lys Ala Ile Gln Glu Gln Leu Ile Ala Asn Val His Ser Asn Asp Asp
                85                  90                  95

Tyr Phe Glu Val Ile Asp Phe Ala Ser Asp Ala Thr Ile Thr Asp Arg
            100                 105                 110

Asn Gly Lys Val Tyr Phe Ala Asp Lys Asp Gly Ser Val Thr Leu Pro
        115                 120                 125
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Thr Gln Pro Val Gln Glu Phe Leu Leu Ser Gly His Val Arg Val Arg
    130                 135                 140

Pro Tyr Lys Glu Lys Pro Ile Gln Asn Gln Ala Lys Ser Val Asp Val
145                 150                 155                 160

Glu Tyr Thr Val Gln Phe Thr Pro Leu Asn Pro Asp Asp Asp Phe Arg
                165                 170                 175

Pro Gly Leu Lys Asp Thr Lys Leu Leu Lys Thr Leu Ala Ile Gly Asp
            180                 185                 190

Thr Ile Thr Ser Gln Glu Leu Leu Ala Gln Ala Gln Ser Ile Leu Asn
        195                 200                 205

Lys Asn His Pro Gly Tyr Thr Ile Tyr Glu Arg Asp Ser Ser Ile Val
    210                 215                 220

Thr His Asp Asn Asp Ile Phe Arg Thr Ile Leu Pro Met Asp Gln Glu
225                 230                 235                 240

Phe Thr Tyr Arg Val Lys Asn Arg Glu Gln Ala Tyr Arg Ile Asn Lys
                245                 250                 255

Lys Ser Gly Leu Asn Glu Glu Ile Asn Asn Thr Asp Leu Ile Ser Glu
            260                 265                 270

Lys Tyr Tyr Val Leu Lys Lys Gly Glu Lys Pro Tyr Asp Pro Phe Asp
        275                 280                 285

Arg Ser His Leu Lys Leu Phe Thr Ile Lys Tyr Val Asp Val Asp Thr
    290                 295                 300

Asn Glu Leu Leu Lys Ser Glu Gln Leu Leu Thr Ala Ser Glu Arg Asn
305                 310                 315                 320

Leu Asp Phe Arg Asp Leu Tyr Asp Pro Arg Asp Lys Ala Lys Leu Leu
                325                 330                 335

Tyr Asn Asn Leu Asp Ala Phe Gly Ile Met Asp Tyr Thr Leu Thr Gly
            340                 345                 350

Lys Val Glu Asp Asn His Asp Asp Thr Asn Arg Ile Ile Thr Val Tyr
        355                 360                 365

Met Gly Lys Arg Pro Glu Gly Glu Asn Ala Ser Tyr His Leu Ala Tyr
    370                 375                 380

Asp Lys Asp Arg Tyr Thr Glu Glu Glu Arg Glu Val Tyr Ser Tyr Leu
385                 390                 395                 400

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCGATATCGC TGGACCTGAG TGG 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGATATCGA CAAAGATGGT TCG 23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCAGATCTA TGAAAAATTA CTTATCTTTT GG 32

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGATCCT CATTATTTGT CGTTAGGGTT ATCAC 35

What is Claimed is:

1. A substantially pure nucleic acid consisting essentially of a nucleotide sequence encoding a polypeptide of amino acids 14 to 414 of SEQ ID NO: 1, wherein said polypeptide binds to plasminogen and said polypeptide does not have an amino acid sequence consisting of amino acids 60 to 414 of SEQ ID NO: 1.

2. The nucleic acid of claim 1, wherein said polypeptide includes the amino acid sequence consisting essentially of amino acids 244 to 352 of SEQ ID NO: 1.

3. The nucleic acid of claim 2, wherein said polypeptide has a sequence selected from the group consisting of amino acids 1 to 352; 120 to 352; 244 to 414; and 244 to 352 of SEQ ID NO: 1.

4. The nucleic acid of claim 2, wherein said polypeptide has the amino acid sequence consisting essentially of amino acids 14 to 414 of SEQ ID NO: 1.

5. An expression vector comprising the nucleotide sequence of claim 1.

6. An expression vector comprising the nucleotide sequence of claim 2.

7. An expression vector comprising the nucleotide sequence of claim 3.

8. An expression vector comprising the nucleotide sequence of claim 4.

9. A host cell transformed with the vector of claim 5.

10. A host cell transformed with the vector of claim 6.

11. A host cell transformed with the vector of claim 7.

12. A host cell transformed with the vector of claim 8.

13. A polypeptide encoded by the nucleic claim 1.

14. A polypeptide encoded by the nucleic claim 2.

15. A polypeptide encoded by the nucleic claim 3.

16. A polypeptide encoded by the nucleic claim 4.

17. A method of detecting plasminogen in a sample of biological material comprising:
    a) contacting said sample with the polypeptide of claim 13 for a period of time sufficient to allow the formation of polypeptide-plasminogen complexes; and
    b) detecting said complexes.

18. A method for assaying streptokinase fragments for antigenicity, comprising contacting said fragment with a monoclonal antibody specific for a distinct epitope of streptokinase and determining whether said fragment binds to said monoclonal antibody.

19. The method of claim 18, further comprising the step of determining the ability of the fragment to activate plasminogen in the presence of said monoclonal antibody.

20. A method for treating myocardial infarction comprising administering the streptokinase fragment of claim 16 to a patient.

21. A monoclonal antibody specific for a distinct epitope of streptokinase.

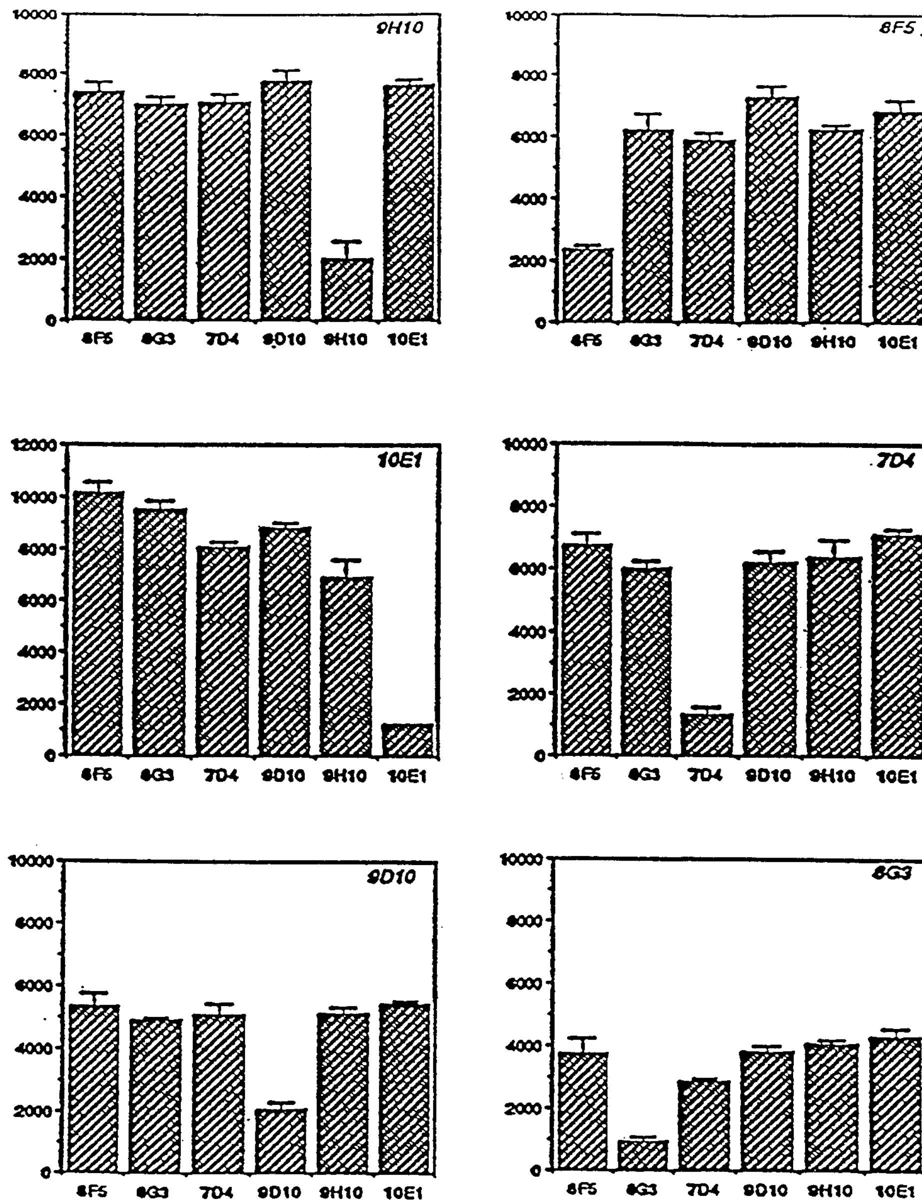

Figure 1

A

```
1                                    10      HincII                     20
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                                    30                                  40
S  V  A  G  T  V  E  G  T  N  Q  D  I  S  L  K  F  F  E  I
                                    50                                  60
D  L  T  S  R  P  A  H  G  G  K  T  E  Q  G  L  S  P  K  S
                                    70                                  80
K  P  F  A  T  D  S  G  A  M  S  H  K  L  E  K  A  D  L  L
                                    90                                 100
K  A  I  Q  E  Q  L  I  A  N  V  H  S  N  D  D  Y  F  E  V
                                   110                                 120
I  D  F  A  S  D  A  T  I  T  D  R  N  G  K  V  Y  F  A  D
                                   130                                 140
K  D  G  S  V  T  L  P  T  Q  P  V  Q  E  F  L  L  S  G  H
                                   150                                 160
V  R  V  R  P  Y  K  E  K  P  I  Q  N  Q  A  K  S  V  D  V
                                   170                                 180
E  Y  T  V  Q  F  T  P  L  N  P  D  D  D  F  R  P  G  L  K
                                   190                                 200
D  T  K  L  L  K  T  L  A  I  G  D  T  I  T  S  Q  E  L  L
                                   210                                 220
A  Q  A  Q  S  I  L  N  K  N  H  P  G  Y  T  I  Y  E  R  D
                                   230                                 240
S  S  I  V  T  H  D  N  D  I  F  R  T  I  L  P  M  D  Q  E
                                   250                                 260
F  T  Y  R  V  K  N  R  E  Q  A  Y  R  I  N  K  K  S  G  L
                                   270                                 280
N  E  E  I  N  N  T  D  L  I  S  E  K  Y  Y  V  L  K  K  G
                                   290                                 300
E  K  P  Y  D  P  F  D  R  S  H  L  K  L  F  T  I  K  Y  V
                                   310                                 320
D  V  D  T  N  E  L  L  K  S  E  Q  L  L  T  A  S  E  R  N
                                   330                                 340
L  D  F  R  D  L  Y  D  P  R  D  K  A  K  L  L  Y  N  N  L
                                   350                                 360
D  A  F  G  I  M  D  Y  T  L  T  G  K  V  E  D  N  H  D  D
                                   370                                 380
T  N  R  I  I  T  V  Y  M  G  K  R  P  E  G  E  N  A  S  Y
                                   390                                 400
H  L  A  Y  D  K  D  R  Y  T  E  E  E  R  E  V  Y  S  Y  L
                                   410            414
R  Y  T  G  T  P  I  P  D  N  P  N  D  K
```

B

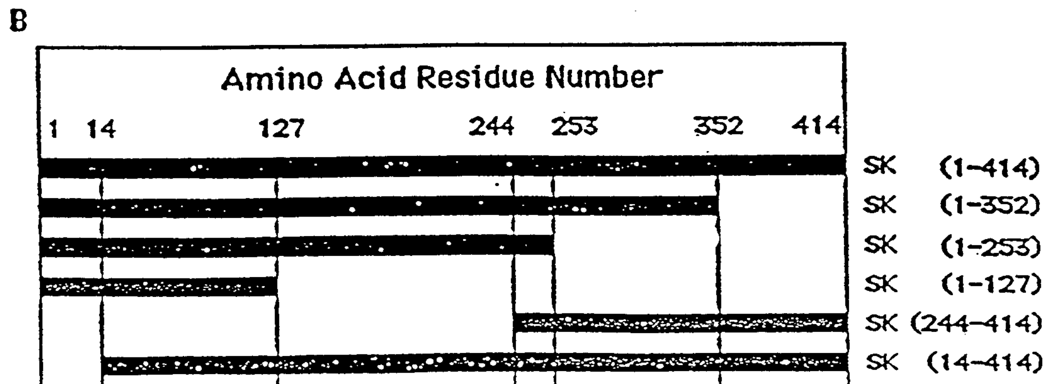
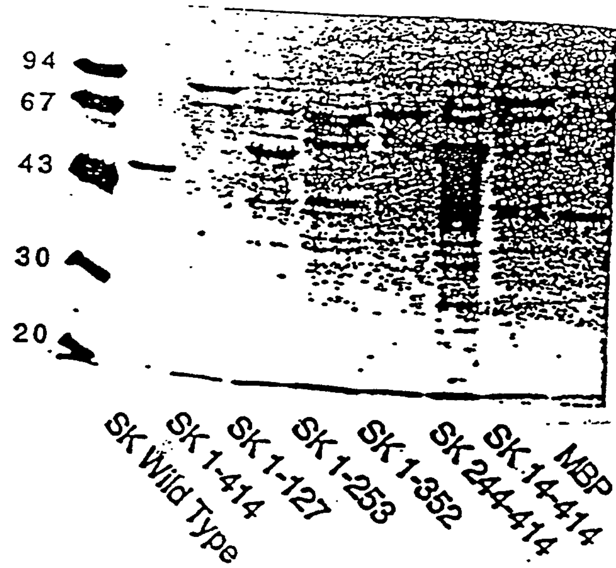


Figure 2



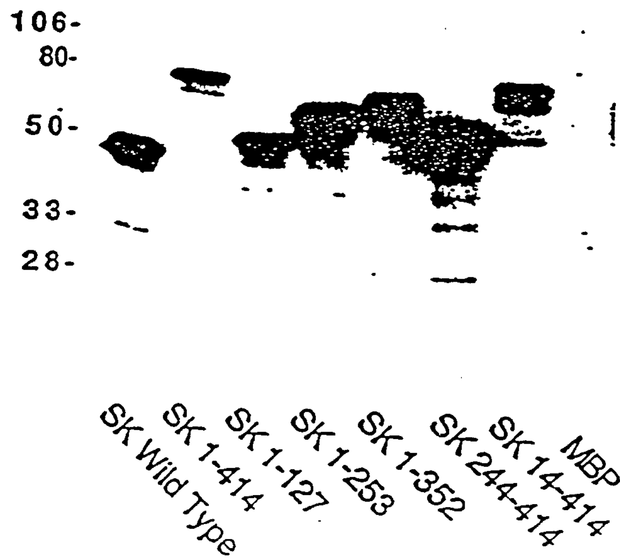


Figure 3

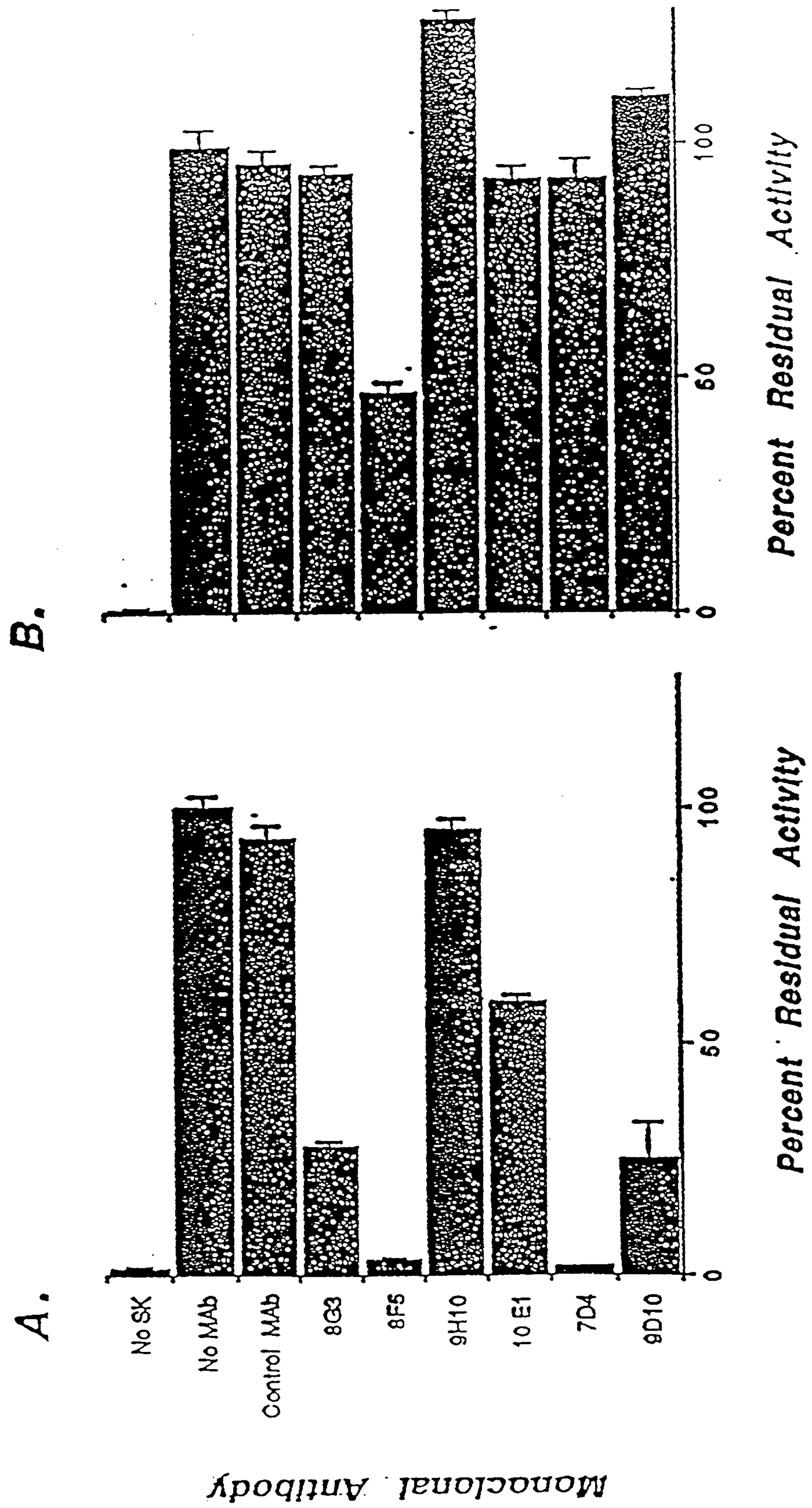


Figure 4

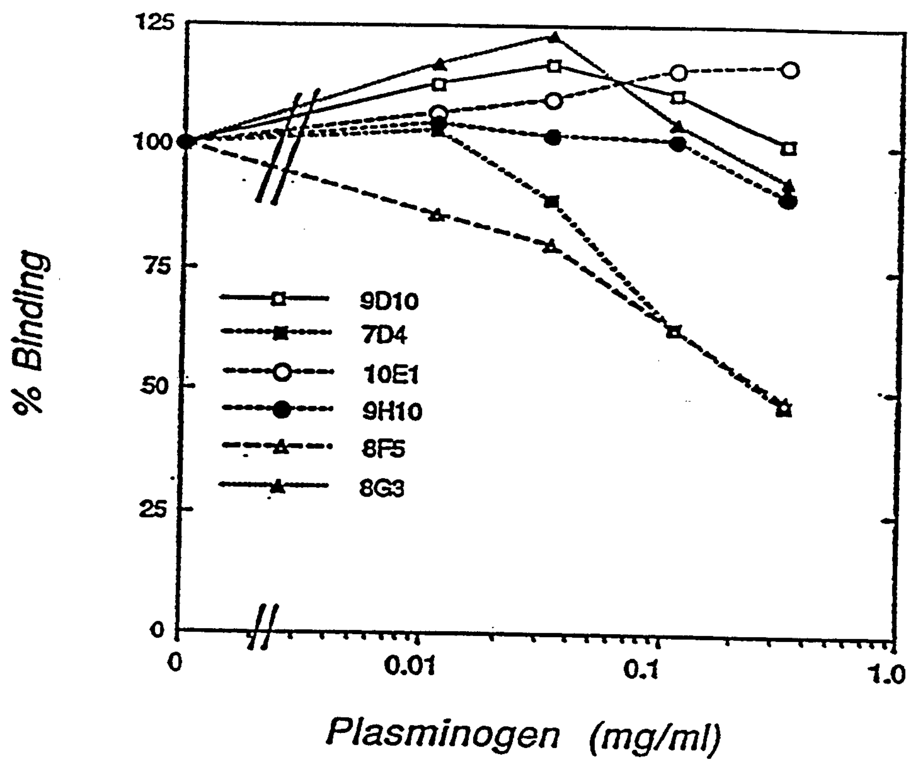


Figure 5

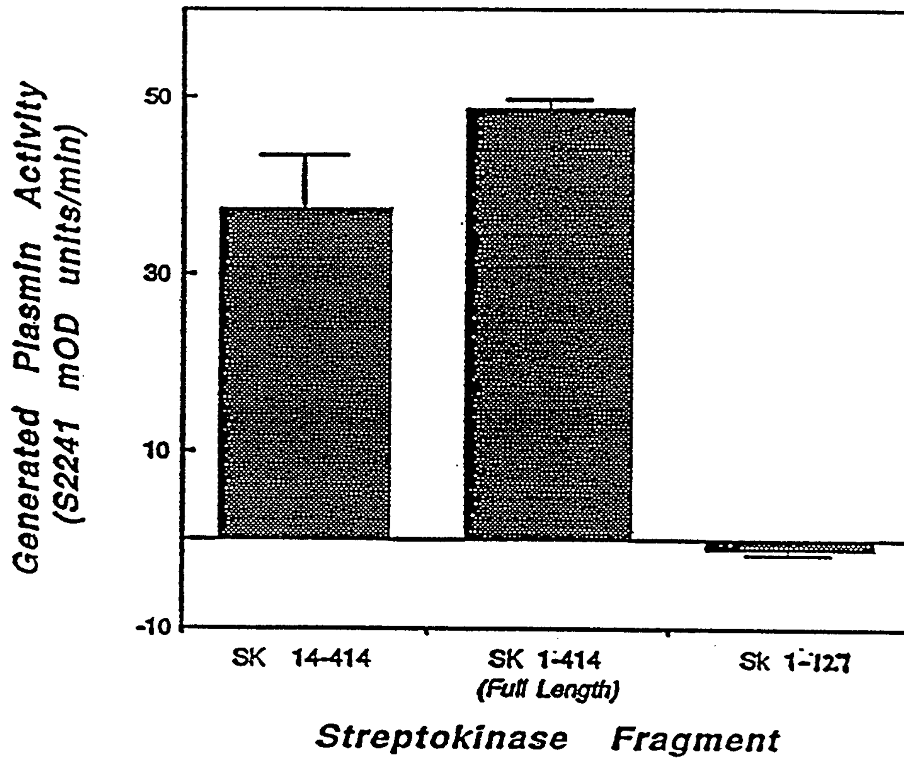


Figure 6

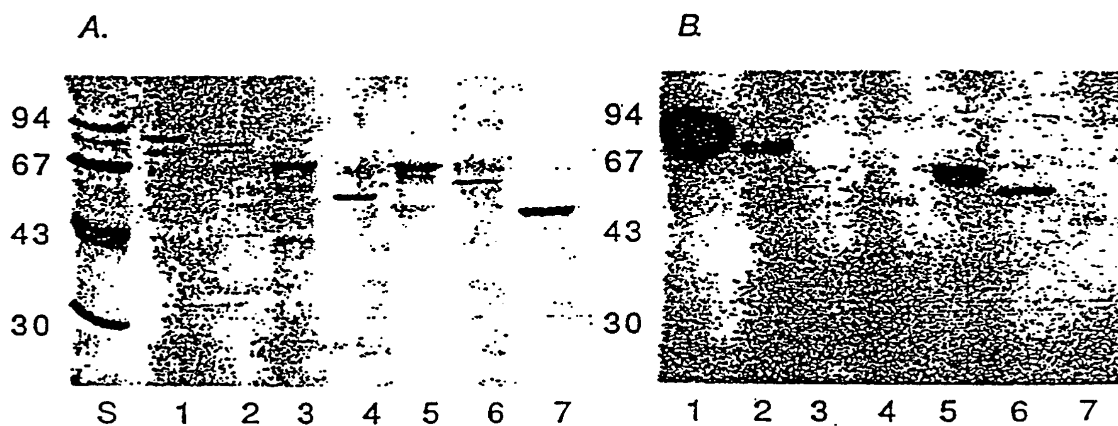


Figure 7

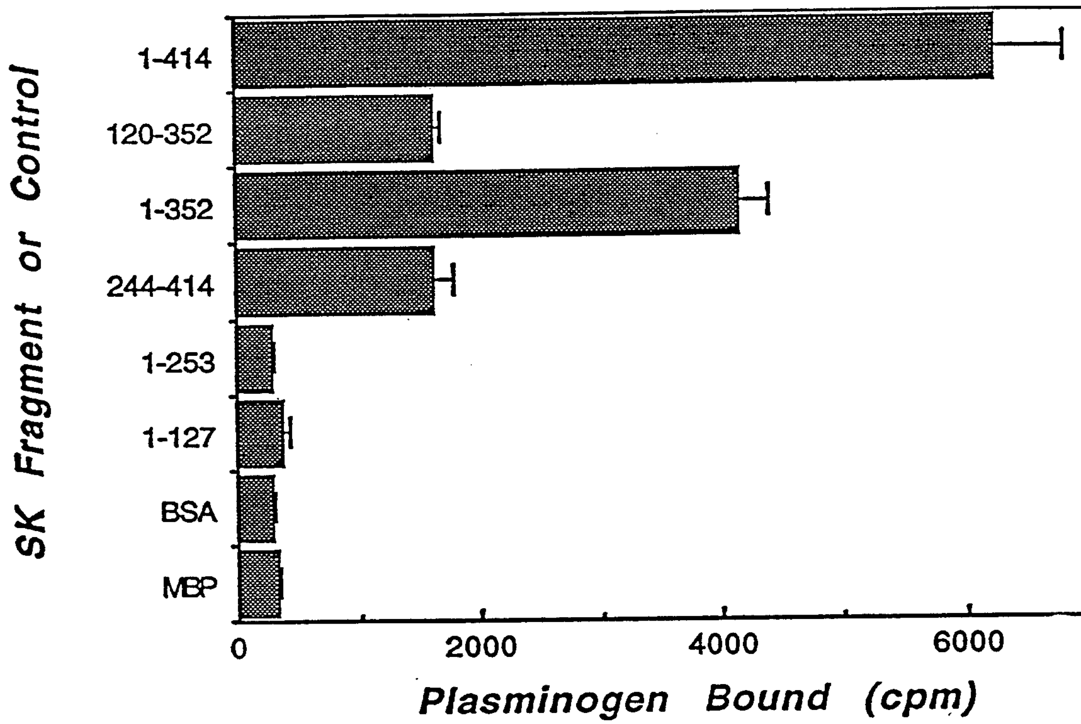


Figure 8

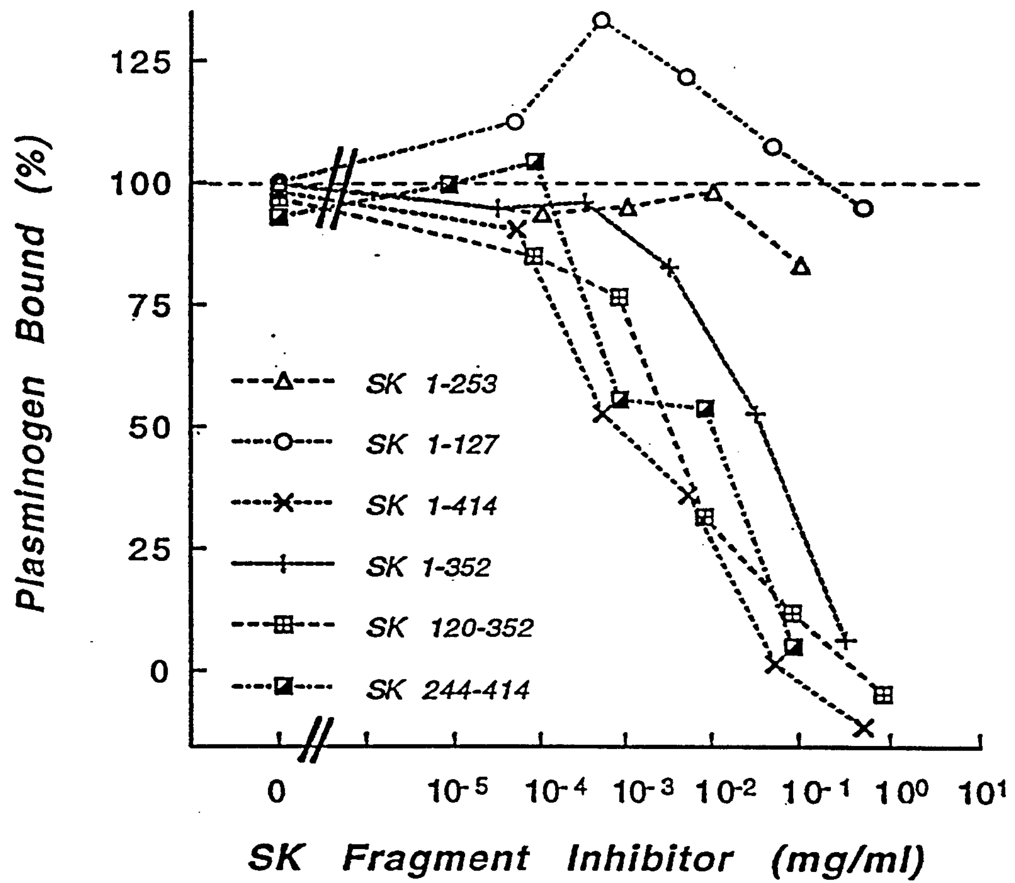


Figure 9

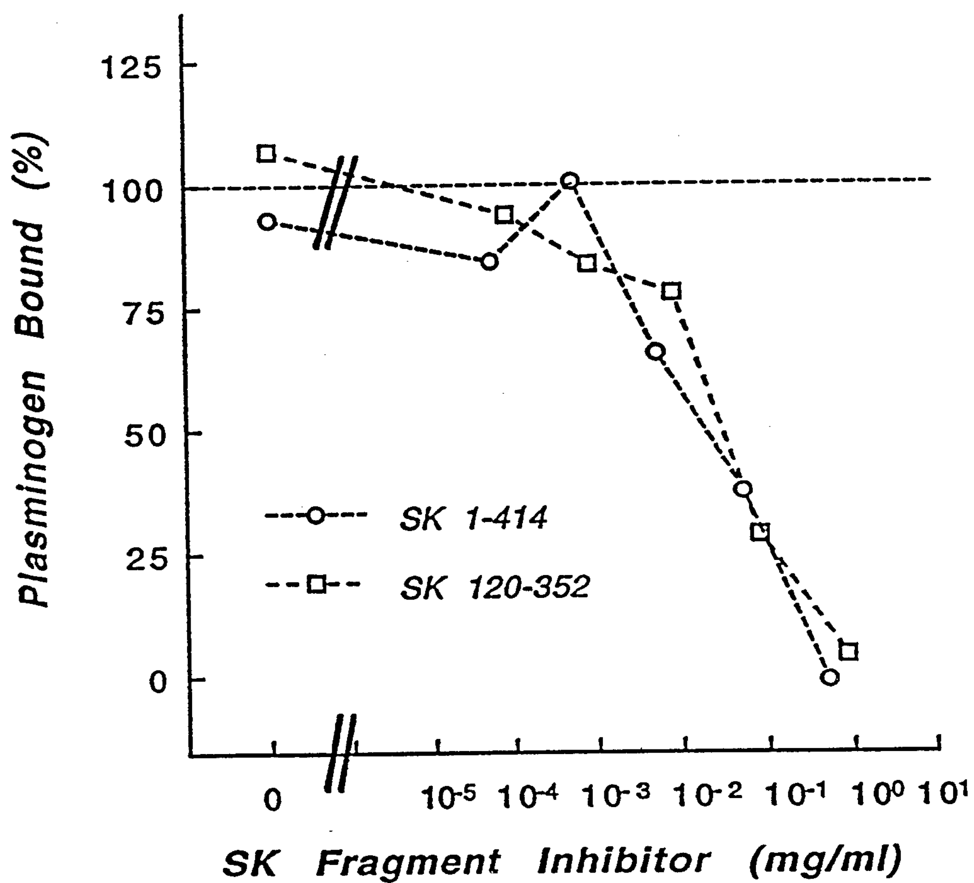


Figure 10

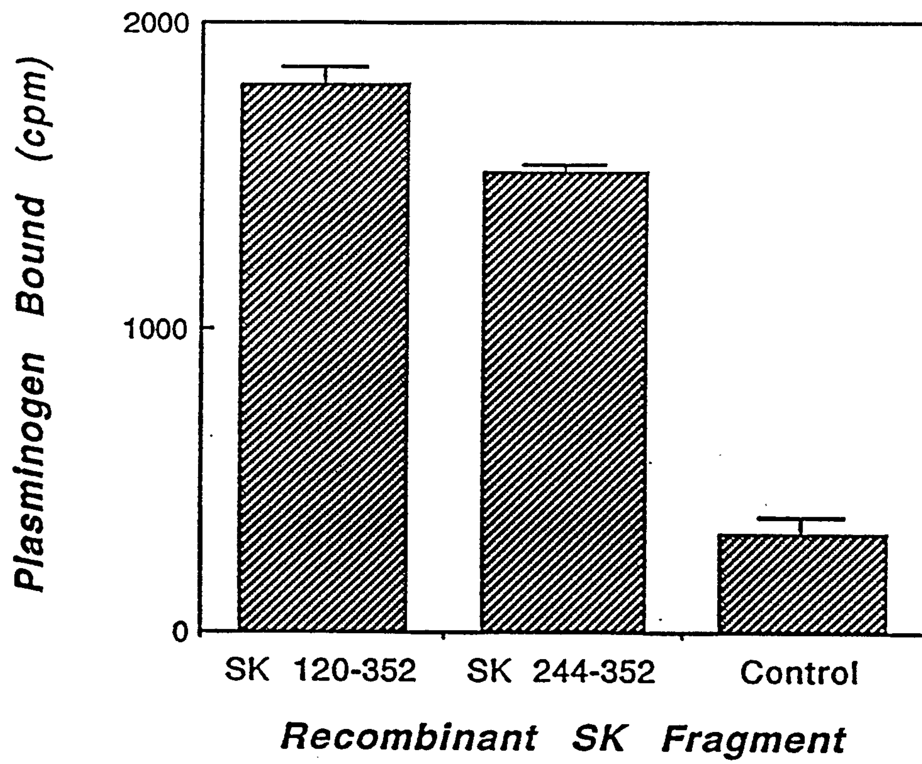


Figure 11

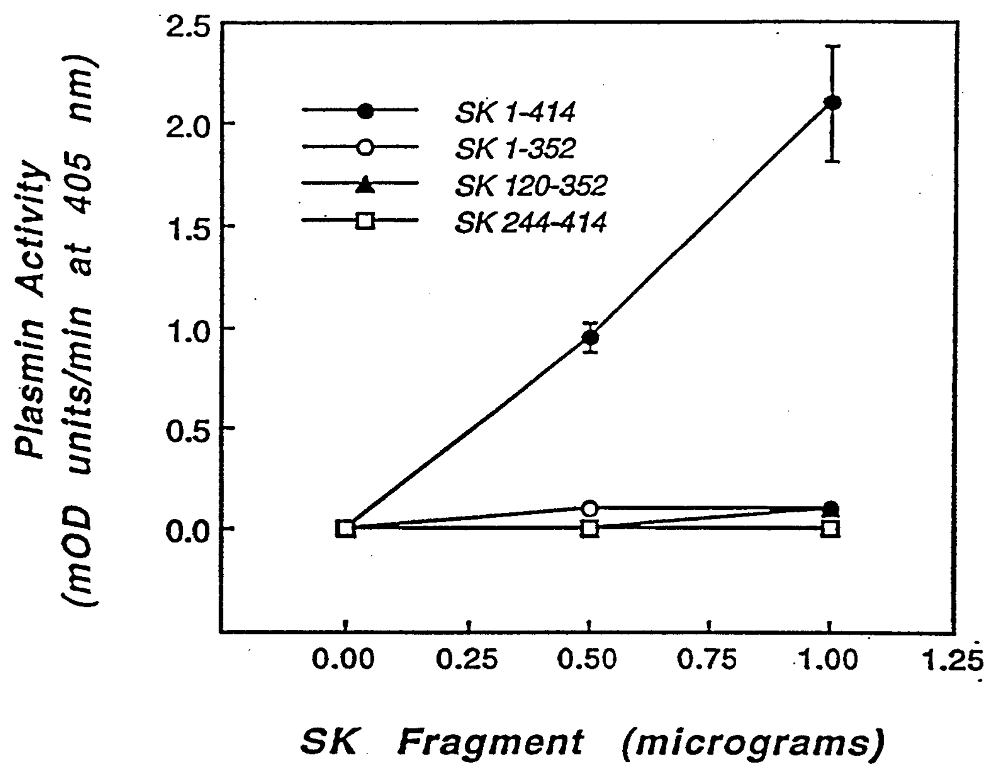


Figure 12

INTERNATIONAL SEARCH REPORT

Inte. ıonal application No.
PCT/US93/09502

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.64; 435/216, 252.3, 320.1; 436/518; 514/12; 530/350, 388.26; 536/23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, PIR, SWISSPROT, GENBANK, UMBL, search terms: sequence, streptokinase, fragment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemistry, Volume 13, Number 10, issued 1974, Brockway et. al., "A characterization of native streptokinase and altered streptokinase isolated from a human plasminogen activator complex", pages 2063-2070, see page 2063.	1-21
X Y	DE, A, 137,325 (Losse et. al.) 29 August 1979, see pages 1 and 5.	13, 16 1-12, 14,15, 17-21
X Y	Gene, Volume 34, issued 1985, Malke et. al., "Nucleotide sequence of the streptokinase gene from Streptococcus equisimilis H46A", pages 357-362, see pages 358 and 360.	1, 4, 13, 16 2, 3, 5-12,14,15,17-21
X,P Y	US, A, 5,187,098 (Malke et. al.) 16 February 1993, see column 3, 5, 6, and 8.	13, 16 1,12,14,15, 17-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
08 December 1993	05 JAN 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KEITH FURMAN, PH.D.
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09502

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	The Journal of Biological Chemistry, Volume 251, Number 13, issued 10 July 1976, Siefring et. al., "Interaction of streptokinase with plasminogen: isolation and characterization of a streptokinase degradation product", pages 3913-3920, see pages 3913 and 3914.	13, 16,17 1-12,14,15, 18-21
X Y	British Heart Journal, Volume 66, issued 1991, Lynch et. al., "Immunoglobulin response to intravenous streptokinase in acute myocardial infarction", pages 139-142, see page 141.	13,16,20,21 1-12,14,15, 17-19
X Y	EP, A, 0,382,696 (Einarsson et. al.) 16 August 1990, see page 2.	13, 16 1-12,14,15, 17-21

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12N 1/00, 15/31,15/58; C07K 15/04, 15/28; G01N 33/53; A61K 37/02, 37/48, 37/547

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.64; 435/216, 252.3, 320.1; 436/518; 514/12; 530/350, 388.26; 536/23.7

CORRECTED VERSION *

PCT

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International Bureau

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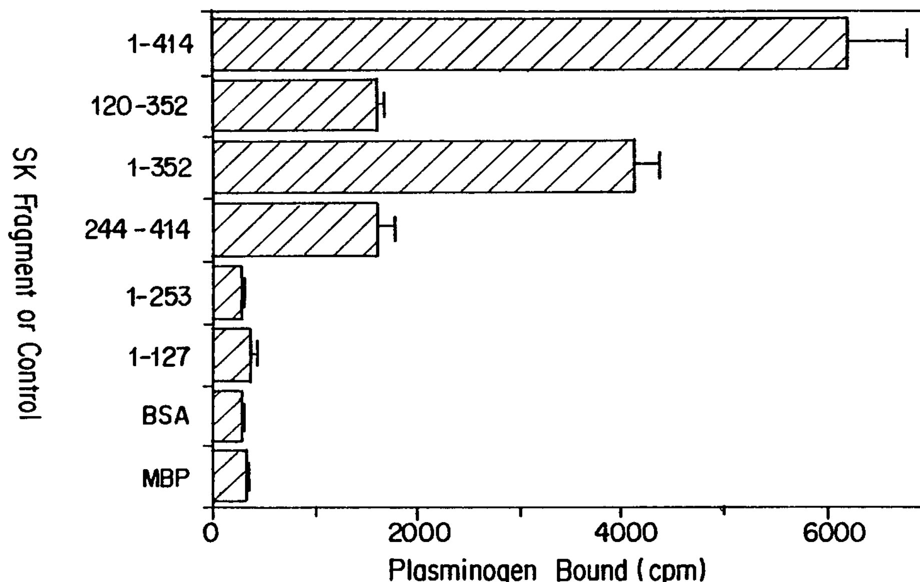
With international search report.

(54) Title: PEPTIDES SPECIFICALLY BINDING TO PLASMINOGEN AND THE DNA ENCODING SUCH PEPTIDES



(57) Abstract

The present invention is directed to nucleic acids which encode polypeptides that bind with specificity to plasminogen and which correspond to regions of streptokinase. The invention is also directed to vectors and hosts which express such nucleic acids and to the polypeptides themselves. The binding of various purified, cleaved recombinant streptokinase fragments to ^{125}I-plasminogen is shown. In addition, the invention is directed to the use of the claimed polypeptides in assays which detect the presence of plasminogen. Streptokinase fragments which retain their ability to activate plasminogen may be used therapeutically.

* (Referred to in PCT Gazette No. 12/1994, Section II)

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Peptides Specifically Binding to Plasminogen And the DNA Encoding Such Peptides

Statement of Government Rights in the Invention

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

The invention is in the field of immunology and molecular biology as related to streptokinase and fragments of streptokinase.

Cross-Reference to Related Applications

This application is a continuation-in-part of United States Patent Application No. 07/956,692 (filed on October 5, 1992) which is incorporated herein by reference.

Background of the Invention

The plasminogen activator streptokinase (hereinafter "SK") is widely used to dissolve blood clots that cause heart attacks. SK is derived from various streptococcus species and differs significantly from the naturally occurring human plasminogen activators urokinase and tissue plasminogen activator (tPA). Urokinase and tPA are serine proteases that cleave a peptide bond in plasminogen to convert it to plasmin, the active enzyme that degrades fibrin. Unlike these human plasminogen activators, SK has no intrinsic enzymatic activity (reviewed in Reddy, K. N., *Enzyme 40*:79-89 (1988)).

The mechanism by which SK and plasminogen form a functional plasminogen activator is not known. After completing their analysis of the primary structure of SK, Jackson and Tang (1982) noted that the 245 amino-terminal residues of SK were homologous with sequences from various serine proteases (*Biochemistry 21*:6620-6625 (1982)). They speculated that SK had

evolved from a serine protease but had lost enzymatic activity when a histidine from its putative active site had been mutated to a glycine.

Similarly, Taylor and Beisswenger (1973) proposed that SK contained the active site of the plasminogen activator (*J. Biol. Chem. 248*:1127-11134
5 (1973)). They observed that SK was proteolytically modified after binding to plasminogen and reported that this modified SK could be isolated and shown to be active. However, their findings were later challenged by Brockway and Castellino (*Biochemistry 13*:2063-2070 (1974)) and have not been confirmed.

The most widely accepted theory of SK's action is that it functions as
10 an allosteric modifier of plasminogen. Two different mechanistic models of this theory have been proposed. One model proposes that SK binds tightly to plasminogen, alters its conformation, and converts it to a plasminogen activator. The modified plasminogen in the SK-plasminogen complex then cleaves the Arg_{560}-Val bond in another plasminogen molecule to form plasmin
15 (Reddy, and Markus, *J. Biol. Chem. 247*:1683-1691 (1972); Schick and Castellino, *Biochemistry 12*:4315-4321 (1973)).

The other mechanistic model, proposed by Kosow, also postulates that SK functions as a catalytic allosteric effector of plasminogen, but by a different mechanism (Kosow, *Biochemistry 14*:4459-4465 (1975)). In this
20 model, SK binds to plasminogen and changes its conformation. This produces an internal active site in plasminogen that "auto"-cleaves an internal bond to produce plasmin. According to this model, SK then dissociates to bind to another plasminogen molecule to repeat the process.

Both of the proposed models postulate that SK acts merely as an
25 allosteric modifier of plasminogen, although they differ in the stability they ascribe to the SK-plasminogen complex and as to whether plasminogen activates itself or other molecules.

Despite speculation concerning the mechanism by which SK exerts its therapeutic effect, the prior art has not defined which regions of the protein
30 bind to plasminogen. The invention disclosed herein relates to peptides which

have amino acid sequences corresponding to specific regions of SK and which bind to plasminogen.

Summary

The invention is directed, *inter alia*, to nucleic acids encoding portions of the streptokinase protein which are capable of binding to plasminogen. Included within the invention are all such nucleic acids encoding polypeptides having sequences corresponding to amino acids 14 to 414 of streptokinase except for the polypeptide consisting of amino acids 60 to 414. Preferably the encoded protein includes the amino acid sequence corresponding to amino acids 244 to 352 of streptokinase. The most preferred embodiments are those nucleic acids encoding proteins with sequences corresponding to amino acids 1 to 352; 120 to 352; 244 to 414; 244 to 352 or 14 to 414 of streptokinase.

The present invention is also directed to vectors and hosts which express the above nucleic acids and to the proteins themselves. The proteins may be used in assays designed to detect plasminogen in biological samples. Proteins which retain their ability to activate plasminogen may be used as a fibrinolytic in the same way that intact streptokinase has been used for decades.

In addition, the invention is directed to a method for assaying streptokinase fragments for antigenicity. This is accomplished by examining the ability of monoclonal antibodies to distinct epitopes of streptokinase to bind peptides with amino acid sequences corresponding to different regions of the streptokinase molecule.

The streptokinase fragment most preferred as a fibrinolytic agent is SK_{14-414}. This fragments exhibits reduced antigenicity relative to intact streptokinase but retains the ability of the intact protein to activate plasminogen.

Brief Description of the Drawings

Figure 1: Competitive epitope binding assays for six different categorical MAbs. Wells of a microtiter plate were coated with the purified MAb indicated in the upper right corner of each graph. Then different purified MAbs (indicated on the abscissa of each graph) were added as inhibitors to the wells in duplicate with ^{125}I-SK. The resulting amount of ^{125}I-SK bound to the immobilized MAb (shown on the ordinate) was determined by gamma-counting.

Figure 2 (A and B): Amino acid sequence of SK and location of peptides. Figure 2, panel A depicts the amino acid sequence of the SK protein (SEQ ID NO: 1). Also indicated by small arrows are the approximate locations of primers used to generate the SK fragments by PCR. The HincII restriction enzyme site is shown. Figure 2, panel B is a schematic illustrating the recombinant SK fragments. The initial and terminal amino acid numbers of each fragment are shown.

Figure 3 (A and B): Electrophoresis and immunoblotting results. Figure 3, panel A, shows the results of the SDS-polyacrylamide (12%, reducing conditions) gel electrophoresis of partially purified MBP-SK fusion proteins, stained with Coomassie Brilliant Blue. Figure 3, panel B, shows the results of an electrophoretic gel which has been immunoblotted with pooled monoclonal antibodies followed by ^{125}I-goat antimouse antibody (panel B). The contents of each lane are shown. Molecular weight markers (kDa) are indicated at left.

Figure 4 (A and B): Effects of MAbs on plasminogen activation by SK. In Figure 4, panel A, MAbs were added prior to formation of SK-plasminogen activator complex. Purified MAbs were premixed with native SK for 20 min. and then added to wells containing plasminogen and a chromogenic substrate (CBS 33.08). The generation of plasmin was monitored by the cleavage of the chromogenic substrate. The percentage residual SK activity was determined as described in the Methods. In Figure 4, panel B, MAbs were added after formation of the SK plasminogen activator

complex. SK and plasminogen were preincubated for 5 minutes and then added to wells of a microtiter plate containing anti-SK MAbs, or a control, inert MAb, plasminogen and chromogenic substrate. The generation of plasmin was monitored and the percent residual activity was determined as described.

Figure 5: Effects of anti-SK MAbs on the binding of SK to plasminogen. Wells of a microtiter plate were coated with purified anti-SK MAbs or no MAb. Various amounts of plasminogen (0 to 1.0 mg/ml), aprotinin (1000 units/ml) and ^{125}I-SK (100,000 cpm) were added to the wells. After 1 hr. of incubation the bells were washed and the amount of bound ^{125}I-SK was determined by gamma counting. The percent inhibition of binding of ^{125}I-SK to the MAbs was computed by reference to wells containing no plasminogen as inhibitor (100% binding) and no MAb (0% binding).

Figure 6: Plasminogen activation by recombinant fragments of streptokinase SK_{1-127} and SK_{14-414}, as well as native SK. Each recombinant fragment or SK was incubated with human plasminogen. Generation of plasmin was monitored by the time-related cleavage of its specific p-nitroanilide substrate, S2251.

Figure 7 (A and B): Binding of electrophoretically separated recombinant MBP-SK proteins to plasminogen. After electrophoresis on 10% SDS-polyacrylamide gels, proteins were stained with Coomassie blue dye (panel A) or electrophoretically transferred to polyvinylidene difluoride membranes and probed with ^{125}I-plasminogen (panel B). The membranes were then subjected to autoradiography to detect bound plasminogen. The lanes contain: lane S, molecular weight standards; lane 1, SK 1-414; lane 2, SK 1-352; lane 3, SK 1-253; lane 4, SK 1-127; lane 5, SK 244-414; lane 6, SK 120-352; and lane 7, MBP. The molecular weights of standards (in kDa) are indicated on the left.

Figure 8: Binding of purified, cleaved recombinant SK fragments to ^{125}I-plasminogen. Wells of a microtiter plate were coated with purified, cleaved SK fragments or control proteins (MBP or bovine serum albumin

(BSA)). After nonspecific binding sites had been blocked with bovine serum albumin, ^{125}I-plasminogen was added to each well. The wells were washed and the bound plasminogen was detected by gamma counting. The data represent the means ± SD of duplicate observations.

5 *Figure 9*: Inhibition of ^{125}I-plasminogen binding to wild-type SK by various purified recombinant SK fragments. Wells of a microtiter plate were coated with wild-type SK. After nonspecific protein binding sites had been blocked, various amounts of recombinant SK fragments, or wild-type SK, were added as inhibitors. Immediately thereafter, ^{125}I-plasminogen was added
10 to the wells. After a 1-hr incubation, the amount of bound ^{125}I-plasminogen was determined by gamma counting. Each point represents the mean of duplicate observations. In this assay 100% plasminogen binding was that occurring in the absence of a recombinant SK inhibitor, and 0% binding was that occurring in the absence of wild-type SK.

15 *Figure 10*: Competitive binding of recombinant SK fragments to plasminogen. Wells of a microtiter plate were coated with SK 244-414. Then the overlapping fragment SK 120-352, or the full-length SK 1-414 (control), was added to the wells as an inhibitor in various concentrations. Subsequently, ^{125}I-plasminogen was added to the wells for 1 h. The wells
20 were washed and the amount of bound plasminogen was determined by gamma counting. Percentage binding was computed as described in Fig. 9.

Figure 11: Binding of plasminogen to SK fragments. Recombinant MBP-SK fragments were immobilized in wells of a microtiter plate. After nonspecific binding sites had been blocked with bovine serum albumin,
25 ^{125}I-plasminogen was added for 1 h. The wells were then washed and gamma counted to detect bound plasminogen. Control wells contained bovine serum albumin only. The data represent the means ± SD of duplicate observations.

Figure 12: Plasminogen activation by various SK fragments. Purified recombinant SK fragments (0-1.0 μg) were mixed with human plasminogen.
30 The generation of plasmin was assayed by continuously monitoring the

cleavage of a specific paranitroanilide substrate of the enzyme. The data represent the means ± SD of duplicate observations.

Definitions

In the description that follows, a number of terms used in biochemistry, recombinant DNA (rDNA) technology and immunology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Gene. A nucleic acid sequence containing a template for a nucleic acid polymerase. The nucleic acid transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA) and, in eukaryotes, is transcribed by RNA polymerase II.

A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of RNA lacking intervening sequences (introns).

Cloning vehicle. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for "cloning vehicle."

Expression vehicle. A vehicle or vector which is similar to a cloning vehicle but is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Expression control sequences will vary depending on whether the

vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

5 Functional Derivative. The present invention pertains to functional derivatives of streptokinase. A "functional derivative" of a SK sequence, either protein or nucleic acid, is a molecule that possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of wild-type SK. The term "functional derivative" is intended to 10 include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, 15 absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art.

20 Fragment. A "fragment" of a molecule such as SK protein or nucleic acid is meant to refer to any portion of a native SK amino acid or nucleotide genetic sequence. Additionally, the term "fragment" includes a chemically synthesized protein fragment of SK.

Variant. A "variant" of a SK protein or nucleic acid is meant to refer 25 to a molecule substantially similar in structure and biological activity to either the native SK molecule, or to a fragment thereof. Thus, provided that two molecules possess a common activity and may substitute for each other, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not 30 identical to that found in the other, or if the amino acid or nucleotide sequence is not identical.

Fusion protein. The term "fusion protein" when applied to the SK fragment of the present invention refers to the product produced from the recombinant gene encoding the SK fragment when it is adjacent to or "fused" to a gene for a carrier protein having no plasminogen activator activity, such that the two genes are "in frame" and yield a single protein, combining the carrier and the SK fragment.

Substantially pure form. The term "substantially pure form" when applied to the SK fragment of the present invention means that the SK fragment is essentially free, i.e., free from any other detectable biological constituents, such as intact host cells with which the SK fragment of the present invention is normally associated.

Immunogenically effective amount. An "immunogenically effective amount" is that amount of SK antigen (SK or a fragment thereof) necessary to induce the production of antibodies which will bind to SK epitopes.

Antigen. The term "antigen" as used in this invention is meant to denote a substance that can induce a detectable immune response to SK when introduced to an animal. Such substances include the SK protein, and fragments thereof.

Epitope. An antibody is said to be capable of binding a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of an antigen which can be recognized and bound by an antibody. An antigen may have one, or more than one epitope. An "antigen" is capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

Antibody. The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable

of binding an antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)).

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Detailed Description of the Invention

A. Construction and Identification of Antibodies to SK

In the following description, reference will be made to various methodologies well-known to those skilled in the art of immunology. Standard reference works setting forth the general principles of immunology include the work of Catty, D. (*Antibodies, A Practical Approach*, Vol. 1, IRL Press, Washington, DC (1988)); Klein, J. (*Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, New York (1982)); Kennett, R., *et al.* in *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, New York (1980)); Campbell, A. ("Monoclonal Antibody Technology," in: *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13 (Burdon, R., *et al.*, eds.), Elsevier, Amsterdam (1984)); and Eisen, H.N., in: *Microbiology*, 3rd Ed. (Davis, B.D., *et al.*, Harper & Row, Philadelphia (1980)).

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Methods for preparing antisera in animals are well-known to those skilled in immunology (see, for example, Chard, *Laboratory Techniques in Biology*, "An Introduction to Radioimmunoassay and Related Techniques," North Holland Publishing Company (1978), pp. 385-396; and *Antibodies, A Practical Handbook*, vols. I and II, D. Catty, ed., IRL Press, Washington, D.C. (1988)). The choice of animal is usually determined by a balance between the facilities available and the likely requirements in terms of volume of the resultant antiserum. A large species such as goat, donkey and horse may be preferred, because of the larger volumes of serum readily obtained. However, it is also possible to use smaller species such as rabbit or guinea pig which often yield higher titer antisera. Usually, subcutaneous injections of the antigenic material (the protein or fragment thereof hapten-carrier protein

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conjugate) are made in order to stimulate the immune system of the animal in which antibodies are to be raised. The detection of appropriate antibodies may be carried out by testing the antisera with appropriately labeled tracer-containing molecules. Fractions that bind tracer-containing molecules are then isolated and further purified if necessary.

Antibodies of the present invention may be prepared by any of a variety of methods. For example, purified SK protein, or a fragment thereof, may be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding to epitopes of the SK protein. If desired, such SK antibodies may be purified from the other polyclonal antibodies by standard protein purification techniques and especially by affinity chromatography with purified SK or fragments thereof.

Monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al., Nature 256*:495 (1975); Kohler *et al., Eur. J. Immunol. 6*:511 (1976); Kohler *et al., Eur. J. Immunol. 6*:292 (1976); Hammerling *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal with SK or a fragment thereof. The splenocytes of immunized animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed; however, it is preferable to employ the parent myeloma cell line (SP_2O), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands, J.R., *et al., Gastroenterology 80*:225-232 (1981). The hybridoma cells obtained are then assayed in order to identify clones which secrete antibodies capable of binding the SK and/or the SK protein antigen. The proliferation of transfected cell lines is potentially more promising than classical myeloma technology, using methods available in the art.

Through application of the above-described methods, additional cell lines capable of producing antibodies which recognize epitopes of the SK protein can be obtained. These antibodies can be used in assays to correlate

SK binding and SK activity with the availability of a particular SK epitope. In this way, information concerning regions of SK which bind to plasminogen may be obtained.

B. Epitope Mapping

5 Once a series of monoclonal antibodies has been produced, the epitopes of SK can be mapped. Initially, each antibody is tested against each other antibody in a competitive binding assay for SK. Those antibodies which effectively inhibit the binding of each other are grouped together and can be said to be specific for a single epitope of SK. Using this method, one MAb
10 from each epitopic group is selected for testing against the representative MAb of each other group to confirm that each is directed against a distinct epitope of SK. Using the deletion mutants, or recombinant SK fragments, described below, each representative MAb and its corresponding epitope is matched to a region on the SK sequence. While starting with large deletions in the SK
15 sequence, it is within the capability of one skilled in the art to produce progressively smaller and more precise deletions to further narrow the site of each epitope, using the antibody binding assay described herein.

C. Cloning of Streptokinase Fragments Which Bind to Plasminogen

20 Based upon the binding experiments set forth in Example 2, it was determined that peptides with sequences corresponding to amino acids 1-352; 120-352; 244-414; and 244-352 of streptokinase (Figure 2, SEQ ID NO: 1) bind to plasminogen. The invention is directed, *inter alia*, to these peptides and to the nucleic acids encoding these peptides.

25 DNAs encoding peptides corresponding to regions of SK which bind to plasminogen may be made synthetically or cloned by any method known in the art (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)). Since both the amino acid and DNA sequences of streptokinase are known, short DNA primers can be synthesized and used

to amplify nucleic acids with the desired sequences from any cell known to contain the streptokinase gene. Procedures for amplifying nucleotide sequences using the polymerase chain reaction (PCR) are well-known in the art (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring
5 Harbor Press (1989)).

A preferred method of cloning the claimed DNAs is set forth in detail in Example 2 and in Example 1, section "c". In this method primers are used to amplify nucleic acid from *Streptococcus equisimilis* using the procedures described by Saiki et al. (*Science 239*:487-491 (1988)) and Reed et al. (*J.*
10 *Immunol. 150:*4407-4415 (1993)). The following pairs of primers were found to be suitable for the amplification and cloning of SK fragments (numbers refer to the amino acid residues; primer restriction endonuclease sites (EcoRV or *Pst*I) are underlined): SK amino terminus (SEQ ID NO: 2), [5′-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxyl terminus (SEQ ID NO: 3),
15 [5′-d(GC CTG CAG TCA TTA TTT GTC GTT AGG)]; SK 244-248 (SEQ ID NO: 4), [5′-d(GC GAT ATC CGT GTT AAA AAT CGG G)]; SK 127-122 (SEQ ID NO: 5), [5′-d(GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; SK 253-248 (SEQ ID NO: 6), [5′-d(GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; SK 352-347 (SEQ ID NO: 7), [5′-d(GC
20 CTG CAG TCA TTA TCC AGT TAA GGT ATA GTC)]; SK 120-124 (SEQ ID NO: 8), [5′-d(GC GAT ATC GAC AAA GAT GGT TCG)]. The amplified DNA may be cloned into a vector in accordance with conventional techniques and sequenced in order to confirm its identity (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

25 D. Expression of Cloned DNAs and Isolation of Peptides

In order to obtain the claimed peptides, the amplified and cloned DNAs can be inserted into an expression vector and the expression vector used to transform an appropriate host cell. A variety of vector/host systems have been described in the literature (Sambrook *et al.*, Molecular Cloning, A Laboratory
30 Manual, Cold Spring Harbor Press (1989)).

Expression vectors typically contain 5' sequences involved in the initiation of transcription and translation. Sequence elements commonly found in expression vectors include promoters, enhancers and upstream activator sequences. Expression vectors may also a encode signal sequence directing 5 the secretion or the compartmentalization of expressed proteins.

The 3'-non-translated region of expression vectors typically contain translation termination sequence elements and, in eukaryotic cells, sequence elements which direct the polyadenylation of mRNA.

The vector containing the nucleic acid encoding the desired SK peptide 10 may be introduced into an appropriate host cell by any of a variety of suitable means, including transfection. After the introduction of the vector, recipient cells are grown in a medium which selects for the growth of vector-containing cells. Expression of the cloned gene sequence results in the production of recombinant protein. This expression can take place in a transient manner, in 15 a continuous manner or in a controlled manner, i.e. expression may be inducible or repressible.

In the preferred embodiment described in Example 2, the cloned DNA is ligated to the pMAL expression vector and expressed as a fusion protein in *Escherichia coli* wherein peptides are fused to a maltose binding protein 20 (MBP; Maina *et al.*, *Gene 74:*365-373 (1988)). The pMAL vector contains the Lac repressor, which allows synthesis to be induced by isopropyl-b-D-thiogalactoside.

Expressed peptides may be purified from the host cell using standard biochemical procedures. Purification is facilitated by the fact that monoclonal 25 antibodies are available which recognize the peptides (see section A above) and because the physical properties of the peptides can be predicted from their known sequence. With respect to the preferred embodiment discussed above, it has been found that SK fusion proteins may be purified by affinity chromatography on an amylose resin, by affinity chromatography on a 30 plasminogen-Sepharose column, or by preparative gel electrophoresis. The

fusion proteins may then be digested with factor Xa and the free peptide isolated.

E. Mutagenesis of SK and SK Peptides

Derivatives of SK or the SK peptides disclosed herein can be prepared by the mutagenesis of DNA. This can be accomplished using one of the mutagenesis procedures known in the art (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

Preparation of derivatives is preferably achieved by site-directed mutagenesis. Site-directed mutagenesis typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, as disclosed by Messing *et al.*, Third Cleveland Symposium on Macromolecules and Recombinant DNA, A. Walton ed., Elsevier, Amsterdam (1981). These phage are commercially available and their use is well known to those skilled in the art. Alternatively, plasmid vectors containing a single-stranded phage origin of replication (Veira *et al., Meth. Enzymol. 153:*3 (1987)) may be employed to obtain single-stranded DNA.

Site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence the DNA sequence which is to be altered. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example by the method of Crea *et al., Proc. Natl. Acad. Sci. (USA) 75:*5765 (1978). The primer is then annealed with the single-stranded vector containing the sequence which is to be altered, and the created vector is incubated with a DNA-polymerizing enzyme such as *E. coli* polymerase I Klenow fragment in an appropriate reaction buffer. The polymerase will complete the synthesis of a mutation-bearing strand. Thus, the second strand will contain the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as JM101 cells, and clones are selected that contain recombinant vectors bearing the mutated sequence.

Whereas the site for introducing a sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at a target region and the newly generated sequences can be 5 screened for the optimal combination of desired activity.

F. Use of the Claimed Nucleic Acids and Peptides

As discussed above, the claimed DNAs can be used to recombinantly produce peptides which have been shown to bind to plasminogen. Using procedures well-known in the art, the DNA molecules can be labeled and used 10 as probes in Northern blots to detect streptokinase mRNA or in Southern blots to detect the presence of the streptokinase gene (see Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1989)).

The peptides may be used in binding assays to detect the presence of plasminogen. Such assays may be performed either using free peptides or 15 peptides which are part of a fusion protein. For example, a cell line may be grown in medium containing radioactively labeled amino acids so that all of the proteins made by the cells are labeled (see Ausubel *et al.*, *Current Protocols in Molecular Biology*, 10.18.1-10.19.1 (1993)). In order to determine if plasminogen is being made, fusion proteins are immobilized by 20 being bound to an appropriate support (e.g. polyvinylidene difluoride membranes, or the wells of a microtiter plate). Nonspecific binding sites on the support are blocked with proteins that do not bind to plasminogen, e.g. by exposing the support to solutions of bovine serum albumin or nonfat milk. The immobilized peptides are then incubated with a cell lysate prepared from 25 the cells grown in the presence of radioactively labeled amino acids. If plasminogen is present in the sample, it will bind to the immobilized peptides and may be detected by washing the support and counting radioactivity.

Alternatively, peptides may be radioactively labeled at tyrosine residues using chloramine T or Iodogen (see Ausubel *et al.*, *Current Protocols in* 30 *Molecular Biology*, 11.16.5-11.16.7 (1993)). Protein samples suspected of

containing plasminogen may be bound to an immobile support such as the wells of a microtiter plate. Nonspecific binding sites may then be blocked as described above and solutions containing the labeled peptides added to the wells. After incubation, the wells may be washed. The amount of plasminogen in the sample would then be determined as the amount of gama radiation remaining in the wells. These types of assays are well-known in the art and are routinely performed by biological laboratories (for a detailed discussion of procedures that can be used, see Ausubel *et al.*, *Current Protocols in Molecular Biology*, 11.2.1-11.2.19 (1993)).

Labeled peptides may also be used to detect the presence of plasminogen *in vivo* by scintigraphy. In this respect, it should be noted that the binding of peptides does not result in the enzymatic activation of plasminogen. Therefore, detection of plasminogen in animals may be accomplished without generating excessive levels of plasmin.

Besides their use in assays designed to detect the presence of plasminogen, the claimed peptides may be used to purify plasminogen. Peptides may be attached to an appropriate chromatography matrix, e.g. Sepharose 4B and then used to purify plasminogen by affinity chromatography. Such procedures are commonly used with monoclonal antibodies and the adaptation of these procedures to use with the claimed peptides is well within the skill of the ordinary biological laboratory (see e.g., Dean *et al.*, *Affinity Chromatography, A Practical Approach*, IRL Press (1986)).

Peptides such as SK_{14-414} which exhibit reduced antigenicity but which maintain the ability to activate plasminogen may be used therapeutically in the same way that intact streptokinase is presently used. Procedures for treating patients with streptokinase are well known in the art (see e.g., Gruppo Italiano per lo Studio della Streptokinase nell'Infarto Miocardico (GISSI), *Lancet 1*:397-402 (1986); ISIS (Second International Study of Infarct Survival) Collaborative Group, *Lancet 2*:349-360 (1988); Spottl and Kaiser, *Thromb. Diath. Haemorrh. 32*:608-616 (1974); and Lynch *et al.*, *Br. Heart J. 66*:139-142 (1991).

Having now generally described this invention, the same will be further described by reference to certain specific examples which are provided herein purposes of illustration only and are not intended to be limiting unless otherwise specified.

5

Example 1

Methods

Monoclonal Antibody Production

Commercially prepared streptokinase (Hoechst Roussel, Somerville, NJ) was further purified by ammonium sulfate precipitation (40%), followed 10 by ion exchange chromatography on DEAE Affigel Blue (Bio-Rad, Richmond, CA) with a NaCl gradient from 0 to 100 mM. The resulting SK was homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K., *Nature 227*:680-685 (1970)).

Female Balb/C mice (Charles River, Wilmington, MA) were 15 immunized subcutaneously with 33 μg of SK in Freund's adjuvant. They were again immunized 2 weeks later and hyperimmunized 3 weeks later with 3 μg of SK intravenously 2 days prior to fusion. After fusion, cells were distributed into ten 96-well microtiter plates. Hybridoma growth was detected in nearly all wells. Hybridoma supernatants were tested for the presence of 20 anti-SK MAbs in a reverse solid-phase assay (Lukacova *et al.*, *Biochemistry 30*:10164-10170 (1991)). Wells of a microtiter plate were coated with goat antimouse antibody (25 μl, 3 μg/ml). After blocking nonspecific binding sites with 1% BSA, 25 μl of hybridoma supernatant was added for 1 hr. The wells were washed, and ^{125}I-SK (100,000 cpm/25 μl) was added. After 1 hr the 25 wells were again washed and the amount of bound SK was determined by gamma counting. The SK was radioiodinated using the Iodogen method (Pierce, Rockford IL) (Fraker *et al.*, *Biochem. Biophys. Res. Commun. 80*:849-857 (1978)) to a specific activity of $\sim 2 \times 10^6$ cpm/μg. Of the 960 wells tested, 13% produced anti-SK antibody capable of capturing greater than 30 2,000 cpm of ^{125}I-SK (2.5 times control binding). Fifty-one hybridomas

produced Ab that consistently captured > 10,000 cpm (> 10 times control values; apparent $K_d \leq 10^{-8}$ M); these were selected for further study. Hybridomas were cloned by limiting dilution and serotyped using Zymed reagents (San Francisco, CA). The isotype of the categorical MAbs (see 5 below) was $Ig_{\gamma 1}K$.

Antibody Binding Assays

a. *Epitope Mapping Assays.* A competitive radioimmunoassay was used to determine whether hybridomas produced Ab that bound to the same epitope on SK. Wells of a microtiter plate were coated with affinity-purified 10 goat antimouse Ab (3 μg/ml) for 90 minutes. Nonspecific protein binding sites were then blocked by incubation with 100 μl of 1% BSA in Tris-buffered saline. The BSA solution was removed and 25 μl of a given hybridoma culture supernatant was added to a series of wells and incubated for 90 minutes. After washing, either culture supernatant from other hybridomas or 15 the same hybridoma (or 1% BSA) was added to these wells as competitors. (The culture supernatant from the same hybridoma was a positive control for inhibition; 1% BSA was a negative control.) Then 25 μl of ^{125}I-SK (~50,000 cpm) was added to the culture supernatants in each well. After 1 hr of incubation the unbound radioactivity was aspirated, the wells were washed, cut 20 and counted. Antibodies were considered to bind to the same epitope if the Ab in the culture supernatant from the second hybridoma inhibited the binding of the ^{125}I-SK to the immobilized Ab (first hybridoma culture supernatant) by more than 50% (typically > 90%). All hybridomas could be classified into categorical or epitopic groups on the basis of these experiments. Based upon 25 this classification, one representative (or categorical) MAb was selected for expansion into ascites. The MAbs were purified by chromatography on protein A-agarose as described (Lukacova *et al.*, *Biochemistry 30*:10164-10170 (1991)). Competition binding studies were repeated using the purified MAbs representing each of different epitopic groups. Plates were coated with 30 20 μg/ml of purified MAb (25 μl) for one hr. The plates were washed and

nonspecific protein binding sites were blocked with 1% BSA for 30 min. Then purified MAbs (diluted to a final concentration of 10 μg/ml) were mixed with ^{125}I-SK and 25 ml of the mixture (50,000 cpm) was added to the wells containing the immobilized MAb. After 60 minutes of incubation, the reaction
5 mixtures were aspirated. The wells were washed and gamma-counted to determine the amount of bound SK.

b. *Binding of MAbs to SK fragments.* Wells of a microtiter plate were coated with purified SK fragments in a concentration of 20 μg/ml or no antigen. After 90 min. the wells were washed and nonspecific binding sites
10 were blocked with 1% BSA. The wells were washed and incubated with culture media for 1 hr. After washing, bound antibody was detected with ^{125}I-goat antimouse antibody. The competition between human plasminogen and the anti-SK MAbs for binding to ^{125}I-SK was measured in a reverse-solid phase assay. Microtiter plates were coated with 20 μg/ml (25 μl) of purified anti-SK
15 MAbs or no MAb for 2 hrs. in duplicate. After being washed, the plates were blocked with 150 μl of 1% BSA for an hour. Then 25 μl of purified human plasminogen (Deutsch and Mertz, *Science 170*:1095-1096 (1970); with concentrations ranging from 0 to 1.0 mg/ml) in 1% BSA and aprotinin (1000 u/ml) were added to the wells followed immediately by ^{125}I-SK (100,000
20 cpm/25 μl). After 1 hr of incubation, the wells were washed, cut and counted. The amount of bound ^{125}I-SK was determined by gamma counting. The percent inhibition of binding of ^{125}I-SK to the anti-SK MAb by different concentrations of plasminogen was computed by reference to the amount bound to the MAb in wells containing no plasminogen (defined as 0%
25 inhibition) and no MAb (defined as 100% inhibition).

Production of Recombinant SK Fragments

Bacterial genomic DNA was prepared from *Streptococcus equisimilis*, strain H46A (Lancefield's Group C, ATCC # 12449) and the SK gene was cloned using PCR (Saiki *et al.*, *Science 233*:1076 (1988)). Two synthetic

oligonucleotide primers were designed based upon a previously published SK nucleotide sequence (Malke *et al.*, *Gene 34*:357-362 (1985)). The upstream primer was designed to recapitulate the codons of the N-terminus leader sequence of SK. The downstream primer corresponded to the antisense sequence of the C-terminus of the SK gene and contained two stop codons. A restriction endonuclease site was incorporated at the 5′ end of each primer in order to facilitate cloning. The oligonucleotide primers used for amplification and cloning of the SK gene were: SK leader peptide (SEQ ID NO: 9), [5′-d(CCC AGA TCT ATG AAA AAT TAC TTA TCT TTT GG)]; SK carboxy terminus (SEQ ID NO: 10), [5′-d(CCC GGA TCC TCA TTA TTT GTC GTT AGG GTT ATC AC)]. The cloned SK DNA was sequenced (Sanger *et al.*, *Proc. Natl. Acad. Sci. USA 74*:5463-5467 (1977)) and found to be identical to the published sequence.

Truncated portions of the SK gene were generated in a similar manner using PCR and specific internal primers. The following primers were used for amplification and cloning of SK fragments (numbers refer to the amino acid residues): SK amino terminus (SEQ ID NO: 2), [5′-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxy terminus (SEQ ID NO: 3), [5′-d (GC CTG CAG TCA TTA TTT GTC GTT AGG)]; $SK_{244\text{-}248}$(SEQ ID NO: 4), [5′-d (GC GAT ATC CGT GTT AAA AAT CGG G)]; $SK_{127\text{-}122}$ (SEQ ID NO: 5), [5′-d (GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; $SK_{253\text{-}248}$ (SEQ ID NO: 6), [5′-d (GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; $SK_{352\text{-}347}$ (SEQ ID NO: 7), [5′-d (GC CTG CAG TCA TTA TCC ACT TAA GGT ATA GTC)]. The primer restriction endonuclease sites are underlined (EcoRV or Pst 1). An internal Hinc II site in the SK gene was also used in order to generate a protein fragment which began with a valine residue, the amino acid in position 14 of full-length SK. After the DNA sequence corresponding to full length SK or the desired SK fragment was cloned, it was ligated into the pMAL expression vector (New England Biolabs, Beverly, MA) and expressed in *E. coli* as a fusion protein (with maltose binding protein, MBP; Maina *et al.*, *Gene 74(2)*:365-73 (1988)). This vector

also contained the Lac repressor, allowing induced synthesis by isopropyl-β-D-thiogalactoside.

The SK fusion proteins were purified by affinity chromatography on an amylose resin as described by the supplier, or by chromatography on a DEAE Affigel Blue agarose (BioRad, Hayward, CA) and the purity was assessed by SDS-PACE (Laemmli, U.K., *Nature 227*:680-685 (1970)). Following SDS-PAGE, SK proteins were stained with Coomassie blue dye or transferred to polyvinylidene difluoride membranes by semi-dry electroblotting as described (Khyse-Anderson, J., *J. Biochem. Biophys Meth. 10*:203-209 (1984)). The membranes were blocked in 5% nonfat milk and probed with pooled anti-SK hybridomas supernatants for 1 hr. After washing 6 times, the membranes were incubated in ^{125}I-goat anti-mouse antibody (1,000,000 cpm) for 1 hr. After additional washing, the membranes were exposed to Kodak X-Omat film (Rochester, NY) at -70^0 C.

Effects of MAbs on SK Activity

Purified MAb (25 μl, 1 mg/ml) was mixed with native SK (25 μl, 1 mg/ml) for 20 minutes at room temperature and then added to wells of a microtiter plate containing 25 μl each of 0.1% BSA in Tris-buffered saline, human plasminogen (100 μg/ml) and CBS 33.08 substrate (AcOH.H-D-NLeuCHA-Arg-pNA, 1.9 mM, Diagnostica Stago, Asnieres-Sur-Seine, France). The rate of plasminogen activation to plasmin was determined by the cleavage of the plasmin substrate CBS 33.08 and subsequent release of the p-nitroanilide product. The rate of p-nitroanilide production was recorded continuously at 405 nm in triplicate for each of these samples in a microtiter plate reader (Thermomax, Molecular Devices, Palo Alto, CA). The rate of p-nitroanilide formation in the presence of a given MAb was compared to the rate of product formation in the presence of a purified, inactive, control antidigoxin MAb (100% activity) or in absence of any SK (0% activity). In other experiments, the effect of the MAbs was studied on the preformed SK-PAC. SK (25 μl, 1 μg/ml) was mixed with human plasminogen (25 μl, 2

μg/ml) for 5 min. and added to wells containing 25 μl each of MAb (1 mg/ml), human plasminogen (1 mg/ml) and CBS substrate. The rate of plasminogen activation was measured as described above.

Streptokinase Activity of Recombinant SK Fragments

5 Recombinant SK fragments SK_{1-127} and SK_{14-414}, as well as native SK, were incubated with human plasminogen. The generation of plasmin was monitored by the time-related cleavage of its specific p-nitroanilide substrate, S2251.

Results

10 From the fused splenocytes of one immunized mouse, 121 hybridomas were found to produce antibody that specifically bound to SK at levels greater than 2.5 times background in a reverse-solid phase radioimmunoassay. Of these, 51 hybridomas consistently 'captured' ^{125}I-SK at levels exceeding ten times control. The epitope binding specificities of these hybridomas were 15 determined by competitive binding experiments. In this assay, an Ab from one hybridoma was immobilized in the wells of a microtiter plate. Then culture supernatant from another hybridoma was added to compete with the immobilized Ab for binding to ^{125}I-SK. Virgin culture media and culture supernatants from the same hybridoma were used as negative and positive 20 controls for inhibition, respectively. Antibodies were considered to bind to the same epitope if the Ab in the culture supernatant inhibited the binding of the ^{125}I-SK to the immobilized Ab by more than 50%. (Typically the inhibition was 90%). These competitive inhibition assays revealed that the hybridomas could be classified into six major epitopic groups. From each group one 25 representative or categorical MAb was selected for further study. These MAbs were expanded into ascites and purified using affinity chromatography. Competitive binding assays were performed with each of these purified MAbs to verify that they bound to different epitopes. Purified MAb was immobilized in wells of a microtiter plates. After blocking nonspecific binding, each of the

purified MAbs was added in duplicate to the wells with ^{125}I-SK. Figure 1 shows that the binding of the immobilized MAb to ^{125}I-SK was inhibited when the same MAb was used as competitor (self-inhibition). But when other MAbs were used as competitor, they caused little or no effect. Thus, these six categorical MAbs appeared to bind to sterically distinct or minimally overlapping epitopes.

To locate the peptide sequences composing the epitopes recognized by these MAbs, SK fragments were generated using PCR and expressed in *E. coli*. The amino acid sequence of native SK is shown in Figure 2A. Above and below the amino acid sequence is the approximate position of the sense and antisense primers used to amplify the DNA coding for the SK fragments. Figure 2B displays the expressed recombinant SK fragments in schematic form. The NH_2-terminal and COOH-terminal amino acid residues of the SK fragments are indicated by number. Figure 3A shows Coomassie blue-stained gels demonstrating the expression of these induced fusion proteins containing the SK fragments. Figure 3B shows the same material subjected to immunoblotting with pooled MAbs against SK. These two figures demonstrate that the major protein product in the induced *E. coli* lysates is the SK fusion protein. All of the SK fusion proteins are of appropriate molecular mass and are immunoreactive with the SK MAbs, while the other component of the fusion protein, MBP, is not reactive.

The binding patterns of the categorical MAbs to these SK fragments were studied in a direct binding assay. The results are tabulated in Table I.

TABLE I. BINDING OF SK MABS TO SK FRAGMENTS

	Monoclonal Antibody*					
SK fragment	**7D4**	**8F5**	**8G3**	**9D10**	**9H10**	**10E1**
SK_{1-127}	-	-	-	+	-	+
SK_{1-253}	+	-	-	+	-	+
SK_{1-352}	+	+	-	+	+	+
SK_{1-414}	+	+	+	+	+	+
SK_{14-414}	+	+	+	-	+	+
$SK_{244-414}$	-	-	+	-	-	-
Epitope**	128-253	120-352	353-414	1-13	120-352	14-127
No. Abs***	6	10	3	5	12	15

*A '+' indicates that the MAb showed significant binding to the SK fragment (typically 10 times background) in a radioimmunoassay. A '-' sign indicates that the MAb did not bind to the SK fragment at levels significantly greater than background.

**The epitope recognized by the antibody is inferred from the pattern of binding indicated in the table, or by additional data presented in Results.

***The total number of antibodies that bind to the same epitope is listed.

MAb 9D10 binds to full length SK (SK_{1-414}) but does not bind to SK_{14-414}, which lacks the first 13 amino terminal amino acids. This would suggest that MAb 9D10 recognizes an epitope largely constructed by the first 13 amino acids of full-length SK. MAb 8G3 binds to $SK_{244-414}$, but not to SK_{1-352}, suggesting that it recognizes an epitope constructed by amino acid residues in the carboxy terminal end of SK within amino acids 353 to 414. Since MAb 10E1 binds to SK_{1-127}, as well as to SK lacking the first 13 amino acids, it appears to recognize an epitope formed by amino acids 14-127. Two MAbs, 8F5 and 9H10, although they bound to different epitopes by

competition assays, had a similar pattern of binding to the SK fragments, as did all of the MAbs in their respective epitopic groups. Neither 8F5 nor 9H10 bound to the overlapping fragments of SK_{1-253} or $SK_{244-414}$, but both MAbs bound to SK_{1-352}. Later experiments (not shown) also revealed that they bound to another SK fragment, $SK_{120-352}$. Thus it would appear that they recognize an epitope constructed by the association of amino acid sequences on both sides of 244-253 region. Finally, MAb 7D4 appears from its pattern of binding to recognize an epitope constructed by amino acids 128-243. Similar direct binding studies were performed with the other 45 MAbs raised against SK. These MAbs bound to SK fragments in a pattern consistent with the epitope group assignment determined by the competitive binding assays described above (data not shown).

Assays were performed to determine the effects of the MAbs on the formation of the SK-PAC and on the rate of plasminogen activation once the activator complex was formed. When purified MAbs were incubated with SK prior to the addition of plasminogen, all but one MAb appeared to inhibit the rate of plasmin generation (Figure 4A). MAbs 7D4 and 8F5 inhibited plasminogen activation by >90%. MAbs 8G3 and 9D10 also inhibited plasminogen activation by 60-75%, while MAb 10E1 had minor effects. Only MAb 9H10 had no apparent effect on SK activity. However, when the MAbs were added after the SK-PAC was allowed to form, only one of the MAbs, 8F5, showed significant inhibition of plasminogen activation (Figure 4B). This suggested that MAb 8F5 interfered with the catalytic function of the SK-PAC, while the other MAbs inhibited SK activity by interfering with the binding of SK to plasminogen during the formation of the SK-PAC. To test this hypothesis, the binding of ^{125}I-SK to plasminogen was measured in the presence and absence of the monoclonal antibodies (Figure 5). This graph shows that MAbs 7D4 and 8F5 were effective inhibitors of the binding of ^{125}I-SK to human plasminogen at plasminogen concentrations up to, and slightly in excess of, the concentration of plasminogen in plasma (210 mg/ml). The

other MAbs showed no significant competition with SK-plasminogen binding at these concentrations.

Figure 6 shows the plasminogen activation by recombinant SK fragments SK_{1-127} and SK_{14-414}, as well as native SK. SK_{1-127} showed no apparent plasminogen activating ability, compared to the control, whereas SK_{14-414} shows preserved plasminogen activating capacity.

Discussion

Streptokinase is now widely used as an effective treatment for dissolving thrombi that cause heart attacks. Although its immunogenicity has been evident since the 1930s, the molecular basis for its antigenicity has not been studied. The present invention provides a panel of 51 murine hybridomas producing Abs against the native protein as probes to begin to analyze the humoral immune response to SK. These 51 hybridomas were initially selected at somatic cell fusion because of their avid binding to ^{125}I-SK in a reverse solid phase assay. Because this type of assay avoids solid-phase immobilization of SK, it should allow all solution-phase epitopes of the protein to be available for binding to the MAbs. Competition binding assays (performed in the same fashion) demonstrated that these MAbs could be grouped into 6 major complementation or epitope groups that are spatially or sterically distinct. The assignment of these MAbs to epitope groups by competition binding studies was confirmed by their characteristic pattern of binding to SK fragments generated by recombinant DNA techniques. The epitopes recognized by most of the categorical MAbs could be localized to a specific SK fragment, as noted in Figure 1. Correlation of the epitope mapping studies with the functional effects of the MAbs on SK activity suggested regions of the SK molecule which may play a critical role in plasminogen binding and the catalytic function-of the SK-PAC.

The anti-SK MAbs studied are by nature a 'selected' sample of the secondary, polyclonal antibody response to SK in these inbred mice. By studying a large number of hybridomas, we sought to derive MAbs which

would be representative of the polyclonal antibody response. In turn, the epitopes identified by these MAbs should be broadly indicative of the major epitopes recognized by the polyclonal antibody response. Previous studies have demonstrated that MAbs can be used advantageously to identify peptide regions important for a molecule's immunogenicity and function (Benjamin *et al.*, *Annu. Rev. Immunol. 2*:67-101 (1984)). Data from these studies provide important functional information which complements the insights gained from studies of the protein's three-dimensional structure. In this study, the epitope pattern of 51 hybridomas was first determined in a competitive liquid phase assay and then confirmed by the pattern of binding of the hybridomas to recombinant fragments of SK. Using these techniques, each of the Abs studied could be assigned to one major epitopic group with little or no overlap between groups (see Figure 1 and Table I). Considering the relative number of MAbs directed to epitopes throughout the molecule (Table I), it would appear that the amino terminal sequence of SK constructs epitopes that may be 'more antigenic' than the epitopes constructed by the carboxy terminal sequence. For example, considering its relatively small size, the amino terminal 13 amino acids of SK appeared to construct, or provide the critical residues for, an epitope recognized by a surprisingly large number of MAbs. The small size of this region suggests that this may be a 'continuous' epitope dependent on the actual linear sequence of amino acid residues in this peptide, or that these residues provide most of the binding energy responsible during contact with the MAbs. Others have also noted that about 10% of the antibodies raised against a protein antigen will bind to a peptide fragment of the protein (Pellequer *et al.*, *Methods Enzymol. 203*:176-201 (1991)). Deletion of these 13 residues appeared to cause only a local change in SK structure since all the other categorical Abs continued to bind to the SK_{14-414} fragment.

An explanation for the antigenicity of the amino terminus may be related to its structure. Although the actual three-dimensional structure of SK is not known, it is likely to be similar to the crystal structure of *Streptomyces*

griseus protease A, as suggested by Jackson and Tang, *Biochemistry 21(26)*:6620-5 (1982)). In the crystal structure of *Streptomyces griseus* protease A, the structure of the first 13 amino acids consists of a two reverse turns connected by a β-strand. This segment is among the most solvent accessible regions in the *Streptomyces griseus* protease A molecule. In addition, there are three charged residues in this 13 amino acid segment of SK (Figure 2A) and one aromatic residue (the only tryptophan in the SK molecule). These residues may be important factors in the antigenicity of this region since it has been shown that both charged residues and aromatic residues play a significant role in binding through salt bridges and hydrophobic interactions (Sheriff *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8075-9 (1987); Amit *et al.*, *Science 233*:747-53(1986)).

The other epitopes in the molecule appear to be more discontinuous in nature. The best examples are the epitopes recognized by 8F5 and 9H10. Neither of these MAbs bound to overlapping fragments representing SK_{1-253} or $SK_{244-414}$. Rather they appeared to recognize an epitope constructed by the interaction of amino acids in the peptide sequences on both sides of the region of 244-253. Finally, though all 51 MAbs could be grouped into 6 major epitopic groups, it is likely that many of these MAbs display differences in their fine binding specificity which could be demonstrated by more subtle changes in the amino acid sequence of SK.

As an additional means of probing the antigenic epitopes of SK we studied the effects of these MAbs on the binding of native SK to plasminogen and on the catalytic activity of SK-PAC. By correlating the MAb binding site data with functional effects of the MAbs we may begin to formulate some hypotheses about the functional regions of SK. For example, MAb 7D4 is a strong inhibitor of the binding of SK to plasminogen but does not appear to affect the catalytic activity of the SK-PAC once it is formed. This suggests that the region recognized by MAb 7D4, amino acids 128-243, may contain a plasminogen binding site. Similarly, MAb 8F5 inhibits the binding of plasminogen to SK as well as inhibiting the catalytic activity of the SK-PAC.

This suggests that amino acids critical to the function of the SK-PAC reside in the region of amino acids 120-352. Further studies using SK fragments can build on these findings to more precisely delineate the structural regions responsible for SK function.

5 Although there is a growing clinical literature on allergic reactions to SK, this report represents the first attempt to dissect the molecular basis of its antigenicity. SK is unique among previously studied model antigens because humans are naturally immunized with it as a result of streptococcal infections. More recently, immunization has occurred as a consequence of the use of SK 10 as a treatment for thrombosis. This natural immunization process facilitates the analysis of the immune response to SK in humans as wells as other mammals.

SK also has other advantages as a model antigen. Its complete amino acid and DNA sequences are known (Malke *et al.*, *Gene 34*:357-362 (1985); 15 Jackson and Tang, *Biochemistry 21(26)*:6620-5 (1982)). It has no carbohydrate chains nor cysteine amino acids; thus recombinant SK produced in bacteria should be antigenically similar to the native molecule. Further, the several naturally occurring variations in SK sequence from different streptococcal strains may allow additional insights into the antigenicity and 20 function of the molecule. Finally, lessons learned from the study of SK in the laboratory may be tested and applied to humans treated with SK as well as those suffering from streptococcal disease.

Example 2

Experimental Procedures

25 Cloning and Expression of SK and SK Peptides

The SK gene was cloned from *Streptococcus equisimilis* by the polymerase chain reaction (PCR) (Saiki et al., *Science 239*:487-491 (1988) as previously described (see Example 1, sections c-e). The cloned SK gene was sequenced (U.S. Biochemicals, Cleveland, Ohio; Sanger *et al.*, *Proc. Natl.*

Acad. Sci. U.S.A. *74*:5463-5497 (1977)) and found to be identical to the published sequence (Malke et al., *Gene* *34*:357-362 (1985)).

Truncated portions of the SK gene were generated in a similar manner using PCR and specific internal primers. The following primers (Genosys, The Woodlands, TX) were used for amplification and cloning of SK fragments (numbers refer to the amino acid residues; primer restriction endonuclease sites (*Eco*RV or *Pst*I) are underlined): SK amino terminus (SEQ ID NO: 2), [5'-d(GC GAT ATC GCT GGA CCT GAG TGG)]; SK carboxyl terminus (SEQ ID NO: 3), [5'-d(GC CTG CAG TCA TTA TTT GTC GTT AGG)]; SK 244-248 (SEQ ID NO: 4), [5'-d(GC GAT ATC CGT GTT AAA AAT CGG G)]; SK 127-122 (SEQ ID NO: 5), [5'-d(GC CTG CAG TCA TTA CAA GGT TAC CGA ACC ATC)]; SK 253-248 (SEQ ID NO: 6), [5'-d(GC CTG CAG TCA TTA CCT ATA AGC TTG TTC CCG)]; SK 352-347 (SEQ ID NO: 7), [5'-d(GC CTG CAG TCA TTA TCC AGT TAA GGT ATA GTC)]; SK 120-124 (SEQ ID NO: 8), [5'-d(GC GAT ATC GAC AAA GAT GGT TCG)].

After the DNA sequence corresponding to full-length SK, or to the desired SK fragment, had been cloned, it was ligated into the pMAL expression vector (New England Biolabs, Beverly, MA) and expressed as a fusion protein in *Escherichia coli* (with maltose binding protein (MBP; Maina et al., *Gene 74,* 365-373 (1988)). This vector also contained the Lac repressor, which allows induced synthesis by isopropyl-b-D-thiogalactoside.

SK fusion proteins were purified by affinity chromatography on an amylose resin (New England Biolabs) as described by the supplier, by affinity chromatography on a plasminogen-Sepharose column, or by preparative gel electrophoresis on a BioRad Model 491 Prep Cell (BioRad, Richmond, CA). The purity of the recombinant SK fusion proteins was assessed by SDS-PAGE (Laemmli, *Nature* *227*:680-685 (1970)).

The SK fusion proteins were cut with factor Xa as described (Maina et al., *Gene* *74*:365-373 (1988)). After purification, the amount of recombinant SK 1-414 was determined using an absorption coefficient of 7.5

for a 1% solution at 280 nm (Reddy, *Enzyme 40,* 79-89 (1988)). The relative concentrations of the cleaved, purified SK fragments were determined by comparative radioimmunoassay with fragment-specific monoclonal antibodies (see Example 1). Briefly, wells of a microtiter plate were coated with various
5 concentrations of SK 1-414 (0, 2.5, 5, 10, 20, and 40 μg/mL). After nonspecific binding sites had been blocked with 1% bovine serum albumin, fragment-specific monoclonal antibodies were added to each well in duplicate. After a 1 h incubation the wells were washed and probed with ^{125}I goat anti-mouse antibody (Cappel Organon Teknika, Durham, NC) for 1 h. After
10 another wash the amount of bound antibody was determined by gamma counting. A standard curve relating antibody binding to SK concentration was derived for each fragment-specific monoclonal antibody. The assay was then repeated with unknown concentrations of purified recombinant SK fragments and appropriate fragment-specific monoclonal antibodies. The concentration
15 of a recombinant SK fragment was determined by reference to the standard curve.

Protein Labeling

Human plasminogen (Sigma, St. Louis, MO) was labeled by the Iodogen method (Pierce, Rockford, IL; Fraker and Speck, *Biochem. Biophys.*
20 *Res. Commun. 80*:849-857 (1978)) to a specific activity of 5500 cpm per ng. Goat anti-mouse antibody was similarly labeled, with specific activities ranging from about 7000 to 10,000 cpm per ng.

Immunoblotting and Ligand Blotting

Purified SK fusion proteins were subjected to electrophoresis on 10%
25 polyacrylamide gels (Laemmli, *Nature 227*:680-685 (1970)). Proteins were stained with Coomassie blue dye or transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by semi-dry electroblotting as described (Kyhse, *J. Biochem. Biophys. Meth. 10*:203-209 (1984)). The membranes were blocked in 5% nonfat milk and probed with ^{125}I-plasminogen (2,000,000

cpm) for 1 h. After the blots had been washed they were subjected to autoradiography.

Plasminogen Binding Assays

The binding to plasminogen of purified recombinant SK fragments and MBP-SK fusion proteins was determined as follows. Wells of a microtiter plate were coated with 25 μL of purified recombinant SK fragment at a concentration of 10 μg/mL for 3 h at room temperature. Control wells were coated with purified MBP (0.5 μg/mL) or no antigen. The wells were washed and nonspecific protein binding sites were blocked with 200 μL of 1% bovine serum albumin for 1 h. The wells were washed again and ^{125}I-plasminogen (1-200,000 cpm) was added to each well for 1 h. The unbound plasminogen was removed, the wells were washed, and the amount of bound plasminogen was determined by gamma counting.

The competitive binding to plasminogen of various recombinant SK fragments was also studied. Wells of a microtiter plate were coated with wild-type SK (25 μL, 1 μg/mL) for 1 h. Nonspecific protein binding sites were blocked by adding 200 μL of 1% bovine serum albumin for 1 h. Then 25 μL of various concentrations of recombinant SK fragment (ranging from 0 to 0.835 mg/ml) or no SK (all diluted in 1% BSA in Tris-buffered saline) was added to wells in duplicate. Immediately thereafter 25 μL of ^{125}I-plasminogen (50,000 cpm) was added to each well. After 1 h of incubation the wells were washed and bound ^{125}I-plasminogen was counted in a gamma counter. Wells coated with 1% bovine serum albumin (Sigma, St. Louis, MO) and no SK were used as negative controls. The percentage of plasminogen binding was determined by computing the fractional binding of ^{125}I-plasminogen to SK in the presence of a given inhibitor in comparison with that occurring in the absence of an inhibitor, after correcting for nonspecific binding.

In another assay we studied the ability of SK 120-352 to inhibit the binding of SK 244-414 to plasminogen. Wells of a microtiter plate were coated in duplicate with 20 μg/ml of SK 244-414 for 2 h. Nonspecific protein

binding sites were blocked with 1% bovine serum albumin (200 μL) for 1 h. Then SK 120-352 (25 μL, various concentrations) or no inhibitor was added to the wells in duplicate. Immediately thereafter ^{125}I-plasminogen (25 μL, 50,000 cpm) was added to the wells. After 1 h of incubation, the wells were
5 washed and the amount of bound plasminogen was measured in a gamma counter. The percentage of plasminogen binding was determined as described above.

Plasminogen Activation

Purified, cleaved SK fragments (50 μL, 0 to 5 μg) or no SK was added
10 to wells of a microtiter plate in duplicate. Next 50 μL of plasmin substrate 2-AcOH.H-D-Nleu-CHA-Arg-pNA (1.9 mM; Diagnostica Stago, Asnières-sur-Seine, France) and 50 μL of plasminogen (1 mg/ml; Deutsch & Mertz, 1970) was added to the wells. For each of these samples, the rate of paranitroanilide production was recorded continuously at 405 nm (in duplicate) in a microtiter
15 plate reader (Thermomax, Molecular Devices, Palo Alto, CA).

RESULTS

SK fragments were generated by PCR (Saiki et al., *Science 239*:487-491 (1988)) and expressed as fusion polypeptides with MBP (at the amino terminus). Fig. 7A shows the purified MBP-SK proteins on a Coomassie blue-
20 stained gel. The expected molecular masses for the purified MBP-SK proteins were: MBP-SK 1-414, 99 kDa; MBP-SK 1-352, 82 kDa; MBP-SK 1-253, 70 kDa; MBP-SK 1-127, 56 kDa; MBP-SK 120-352, 69 kDa; MBP-SK 244-414, 62 kDa; and MBP-LacZ (no SK), 53 kDa. All the expressed proteins were of the expected molecular masses, except for the MBP-SK 1-414
25 protein, which consistently migrated slightly farther than expected.

In order to determine which MBP-SK fragments bound to human plasminogen, ligand blotting studies were performed. MBP-SK fragments were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then probed with

^{125}I-human plasminogen. Fig. 7B shows that full-length SK (SK 1-414) bound plasminogen, as did the fragments SK 1-352, SK 244-414, and SK 120-352. Fragments SK 1-127 and SK 1-253, and MBP alone did not show significant binding to plasminogen.

5 In order to determine whether the binding of MBP-SK fragments SK 1-127 or SK 1-253 was affected by the presence of the MBP moiety, the fusion proteins were cleaved with factor Xa. After purification, the binding of the cleaved SK fragments (i.e., without MBP) was studied in a solid-phase radioligand assay. Fig. 8 shows that, compared with the negative control 10 proteins (bovine serum albumin and MBP), SK fragments 1-414, 1-352, 120-352, and 244-414 all showed significant binding to plasminogen, but SK 1-127 and 1-253 did not. These results confirmed the previous ligand blotting results with the MBP-SK fusion proteins and argued that the MBP moiety did not prevent binding of the SK fragments to plasminogen.

15 A series of inhibition experiments with purified, cleaved SK fragments was performed to determine the location of plasminogen binding sites. In this series, the ability of SK fragments to interfere with the binding of wild-type SK to ^{125}I-plasminogen was examined. Fig. 9 shows that SK 1-414 fully inhibited the binding of wild-type SK. In addition, recombinant SK fragments 20 1-352, 120-352, and 244-414 caused complete (i.e., $\geq$ 90%) inhibition of plasminogen binding to wild-type SK. However, recombinant SK fragments 1-127 and 1-253 did not show significant inhibition at these concentrations. Because recombinant SK fragments 1-352, 120-352, and 244-414 completely inhibited the binding of plasminogen to wild-type SK, it was inferred that each 25 of the three fragments contained the same plasminogen binding site(s) as native SK.

The smallest fragment that effectively bound plasminogen in these experiments was SK 244-414. Since the next smallest fragment that also bound plasminogen was SK 120-352, it was postulated that the plasminogen 30 binding site(s) were in a region of SK shared by these two fragments, i.e. SK 244-352. To test this hypothesis the ability of SK 120-352 to completely

inhibit the binding of plasminogen to SK 244-414 was examined. Fig. 10 shows that SK 120-352, like full-length SK 1-414, completely inhibited the binding of plasminogen to SK 244-414. This suggested that the region shared by these two fragments, SK 244-352, contained most of the residues necessary for plasminogen binding in these assays.

To investigate this possibility, the SK 244-352 fragment was expressed using the same vector. Fig. 11 compares the binding of SK 244-352, SK 120-352, or no antigen to ^{125}I-plasminogen in a radioimmunoassay. In comparison with control wells, wells containing SK 244-352 and SK 120-352 showed significant binding to plasminogen, confirming that the smaller fragment contained the plasminogen binding site(s).

Enzymatic assays were performed to determine whether binding of an SK fragment to plasminogen was sufficient to generate a functional plasminogen activator complex (Figure 12). The original cleaved SK fragments were incubated with human plasminogen and the generation of plasmin was monitored by recording the rate of cleavage of a chromogenic substrate. In this assay, SK 1-414 showed a dose-dependent activation of plasminogen. However, the fragments that bound to plasminogen, SK 1-352, SK 244-414, and SK 120-352, showed no discernible ability to activate plasminogen.

DISCUSSION

Despite its long and extensive use as a therapeutic agent, the molecular mechanisms by which SK acts to form a plasminogen activator remain unknown. Unlike tPA and urokinase, two other widely used plasminogen activators that are serine proteases, SK forms a tight, noncovalent complex with plasminogen to construct a functional plasminogen activator ($K_d = 5 \times 10^{-10}$ M; Wiman, *Thromb. Res. 17*:143-152 (1980)). In Example 1, it was reported that monoclonal antibodies that bind to the regions of SK spanning amino acid residues 120 to 352 inhibited the binding of SK to plasminogen (see also, Reed et al., *J. Immunol. 150*:4407-4415 (1993)). This observation

suggested that the 120-352 region of SK could be involved in plasminogen binding.

In the present experiments, binding studies with SK deletion mutants showed that SK 1-352, SK 120-352, and SK 244-414 also bound to plasminogen and fully inhibited the binding of wild-type SK to plasminogen. This implied that these three fragments contained all the moderate- to high-affinity plasminogen binding sites. Mutual inhibition studies showed that the smallest of the overlapping fragments, SK 120-352 and SK 244-414, competed with each other for binding to plasminogen. Thus, the region of SK shared by the two fragments, SK 244-352, appeared to contain the plasminogen binding site(s). To confirm this observation, SK 244-352 was expressed and found to bind to plasminogen.

The experiments herein suggest that the SK 244-352 region is the primary moderate- to high-affinity binding region in SK for plasminogen. The assays used could have detected a binding interaction in which the binding affinity of the SK fragments for plasminogen was greater than about 10^5 M^{-1} (Eshar, in *Hybridoma Technology in the Biosciences and Medicine* (Springer, T. A., Ed.) pp 3-41, Plenum, New York (1985)). No significant direct binding of plasminogen to the regions of SK spanned by the fragments SK 1-127 or 1-253 was detected. In addition, recombinant SK fragments sharing the common region 244-352 were able to completely inhibit the binding of wild-type SK to plasminogen, arguing that another section of SK did not contribute significantly to plasminogen binding.

Nevertheless, there were notable differences in the avidity with which recombinant SK fragments 1-352, 120-352, and 244-414 bound to plasminogen (Fig. 8). These differences suggest that there may be a second binding site in SK which was induced by the initial binding of the 244-352 region to plasminogen. Alternatively, amino acid sequences outside the 244-352 region may be necessary for obtaining an SK conformation optimal for plasminogen binding. Because deletion mutants will not necessarily assume the optimal conformation for binding to plasminogen, no attempt was made to precisely

measure their individual affinities for plasminogen. Rather, by determining which fragments bound plasminogen, a region of SK was identified that may be more intensively explored in the future by site-directed mutagenesis.

There have been surprisingly few studies correlating the structure and function of SK. A few studies have examined the activity of SK proteolytic fragments. For example, Klessen et al. (*Mol. Gen. Genet. 212*:295-300 (1988)) showed that deletion of the first 15 amino acid residues in SK did not affect its activity. Similarly, Brockway and Castellino noted that SK lacking the first 59 amino acids was still a functional plasminogen activator (*Biochemistry 13*:2063-2070 (1974)). Jackson et al. (*Biochemistry 25*:108-114 (1986)) demonstrated that deletion of the carboxyl terminal amino acids (beyond residue 383) did not abolish function. Jackson and Tang noted that there was amino acid sequence homology between the amino terminal and carboxyl terminal halves of SK (*Biochemistry 21*:6620-6625 (1982)). They suggested that SK may have two similar domains, each of which binds plasminogen (Jackson et al., in *The Regulation of Coagulation* (Mann, K. G., & Taylor, F. B., Jr, eds.) pp 515-520, Elsevier, North Holland (1980)), and that these domains may have arisen by gene duplication (Jackson and Tang, *Biochemistry 21*:6620-6625 (1982)).

The results disclosed herein do not support this model, since the amino terminal half of SK, as represented by SK 1-253, did not bind to plasminogen. Also, the hypothesis of gene duplication has not been supported by analysis of the SK DNA sequence (Malke et al., *Gene 34*:357-362 (1985)). The present results indicate that the mere binding of plasminogen to SK is not sufficient for the construction of a functional SK-plasminogen activator complex. Unless plasminogen binds to some other occult, low-affinity, or inducible binding site in SK, it appears unlikely that SK merely functions as an allosteric activator or effector.

A more likely hypothesis is that SK binds to plasminogen and contributes amino acids to the active site of the plasminogen activator complex. This possibility was first suggested by Jackson and Tang

(*Biochemistry 21*:6620-6625 (1982)), who noted that SK had some homology with serine proteases. By comparing the amino acid sequence of SK with those of other serine proteases, Jackson and Tang theorized that the usual active site residue histidine was replaced by a glycine at position 24 in SK.

5 To investigate this hypothesis, Lee et al. mutated glycine-24 to a histidine, a glutamine, or an alanine residue (*Biochem. Biophys. Res. Commun. 165*:1085-1090 (1989)). Rather than restore the "lost" serine protease activity of SK, the glycine-to-histidine and glycine-to-glutamine mutations caused almost complete loss of activity, but had no effect on 10 plasminogen binding. Interestingly, the glycine-to-alanine mutation had no effect on activity or plasminogen binding. Although these observations about site-specific mutations do not support assertions that SK has evolved from a serine protease, they do indicate that minor mutations, which do not affect plasminogen binding, have profound effects on the function of SK as a 15 plasminogen activator. Therefore, these observations support the present finding that the mere binding of SK to plasminogen is insufficient for formation of a functional SK-plasminogen activator complex.

All references cited herein are fully incorporated by reference. Having now fully described the invention, it will be understood by those of skill in the 20 art that the scope may be performed within a wide and equivalent range of conditions, parameters and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Reed, Guy L.

(ii) TITLE OF INVENTION: Peptides Specifically Binding to Plasminogen And the DNA Encoding Such Peptides

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: (to be assigned)
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sanzo, Michael A.
(B) REGISTRATION NUMBER: 36,912
(C) REFERENCE/DOCKET NUMBER: 0609.3570001

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 414 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ile Ala Gly Pro Glu Trp Leu Leu Asp Arg Pro Ser Val Asn Asn Ser
1               5                   10                  15

Gln Leu Val Val Ser Val Ala Gly Thr Val Glu Gly Thr Asn Gln Asp
            20                  25                  30

Ile Ser Leu Lys Phe Phe Glu Ile Asp Leu Thr Ser Arg Pro Ala His
        35                  40                  45

Gly Gly Lys Thr Glu Gln Gly Leu Ser Pro Lys Ser Lys Pro Phe Ala
    50                  55                  60

Thr Asp Ser Gly Ala Met Ser His Lys Leu Glu Lys Ala Asp Leu Leu
65                  70                  75                  80

Lys Ala Ile Gln Glu Gln Leu Ile Ala Asn Val His Ser Asn Asp Asp
                85                  90                  95

Tyr Phe Glu Val Ile Asp Phe Ala Ser Asp Ala Thr Ile Thr Asp Arg
            100                 105                 110

Asn Gly Lys Val Tyr Phe Ala Asp Lys Asp Gly Ser Val Thr Leu Pro
        115                 120                 125
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Thr Gln Pro Val Gln Glu Phe Leu Leu Ser Gly His Val Arg Val Arg
    130                 135                 140

Pro Tyr Lys Glu Lys Pro Ile Gln Asn Gln Ala Lys Ser Val Asp Val
145                 150                 155                 160

Glu Tyr Thr Val Gln Phe Thr Pro Leu Asn Pro Asp Asp Asp Phe Arg
                165                 170                 175

Pro Gly Leu Lys Asp Thr Lys Leu Leu Lys Thr Leu Ala Ile Gly Asp
            180                 185                 190

Thr Ile Thr Ser Gln Glu Leu Leu Ala Gln Ala Gln Ser Ile Leu Asn
        195                 200                 205

Lys Asn His Pro Gly Tyr Thr Ile Tyr Glu Arg Asp Ser Ser Ile Val
    210                 215                 220

Thr His Asp Asn Asp Ile Phe Arg Thr Ile Leu Pro Met Asp Gln Glu
225                 230                 235                 240

Phe Thr Tyr Arg Val Lys Asn Arg Glu Gln Ala Tyr Arg Ile Asn Lys
                245                 250                 255

Lys Ser Gly Leu Asn Glu Glu Ile Asn Asn Thr Asp Leu Ile Ser Glu
            260                 265                 270

Lys Tyr Tyr Val Leu Lys Lys Gly Glu Lys Pro Tyr Asp Pro Phe Asp
        275                 280                 285

Arg Ser His Leu Lys Leu Phe Thr Ile Lys Tyr Val Asp Val Asp Thr
    290                 295                 300

Asn Glu Leu Leu Lys Ser Glu Gln Leu Leu Thr Ala Ser Glu Arg Asn
305                 310                 315                 320

Leu Asp Phe Arg Asp Leu Tyr Asp Pro Arg Asp Lys Ala Lys Leu Leu
                325                 330                 335

Tyr Asn Asn Leu Asp Ala Phe Gly Ile Met Asp Tyr Thr Leu Thr Gly
            340                 345                 350

Lys Val Glu Asp Asn His Asp Asp Thr Asn Arg Ile Ile Thr Val Tyr
        355                 360                 365

Met Gly Lys Arg Pro Glu Gly Glu Asn Ala Ser Tyr His Leu Ala Tyr
    370                 375                 380

Asp Lys Asp Arg Tyr Thr Glu Glu Glu Arg Glu Val Tyr Ser Tyr Leu
385                 390                 395                 400

Arg Tyr Thr Gly Thr Pro Ile Pro Asp Asn Pro Asn Asp Lys
                405                 410
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
GCGATATCGC TGGACCTGAG TGG                                          23
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCTGCAGTC ATTATTTGTC GTTAGG 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGATATCCG TGTTAAAAAT CGGG 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCTGCAGTC ATTACAAGGT TACCGAACCA TC 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCTGCAGTC ATTACCTATA AGCTTGTTCC CG 32

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCCTGCAGTC ATTATCCACT TAAGGTATAG TC 32

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGATATCGA CAAAGATGGT TCG 23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCAGATCTA TGAAAAATTA CTTATCTTTT GG 32

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCGGATCCT CATTATTTGT CGTTAGGGTT ATCAC 35

What is Claimed is:

1. A substantially pure nucleic acid consisting essentially of a nucleotide sequence encoding a polypeptide of amino acids 14 to 414 of SEQ ID NO: 1, wherein said polypeptide binds to plasminogen and said polypeptide does not have an amino acid sequence consisting of amino acids 60 to 414 of SEQ ID NO: 1.

2. The nucleic acid of claim 1, wherein said polypeptide includes the amino acid sequence consisting essentially of amino acids 244 to 352 of SEQ ID NO: 1.

3. The nucleic acid of claim 2, wherein said polypeptide has a sequence selected from the group consisting of amino acids 1 to 352; 120 to 352; 244 to 414; and 244 to 352 of SEQ ID NO: 1.

4. The nucleic acid of claim 2, wherein said polypeptide has the amino acid sequence consisting essentially of amino acids 14 to 414 of SEQ ID NO: 1.

5. An expression vector comprising the nucleotide sequence of claim 1.

6. An expression vector comprising the nucleotide sequence of claim 2.

7. An expression vector comprising the nucleotide sequence of claim 3.

8. An expression vector comprising the nucleotide sequence of claim 4.

9. A host cell transformed with the vector of claim 5.

10. A host cell transformed with the vector of claim 6.

11. A host cell transformed with the vector of claim 7.

12. A host cell transformed with the vector of claim 8.

13. A polypeptide encoded by the nucleic claim 1.

14. A polypeptide encoded by the nucleic claim 2.

15. A polypeptide encoded by the nucleic claim 3.

16. A polypeptide encoded by the nucleic claim 4.

17. A method of detecting plasminogen in a sample of biological material comprising:
    a) contacting said sample with the polypeptide of claim 13 for a period of time sufficient to allow the formation of polypeptide-plasminogen complexes; and
    b) detecting said complexes.

18. A method for assaying streptokinase fragments for antigenicity, comprising contacting said fragment with a monoclonal antibody specific for a distinct epitope of streptokinase and determining whether said fragment binds to said monoclonal antibody.

19. The method of claim 18, further comprising the step of determining the ability of the fragment to activate plasminogen in the presence of said monoclonal antibody.

20. A method for treating myocardial infarction comprising administering the streptokinase fragment of claim 16 to a patient.

21. A monoclonal antibody specific for a distinct epitope of streptokinase.

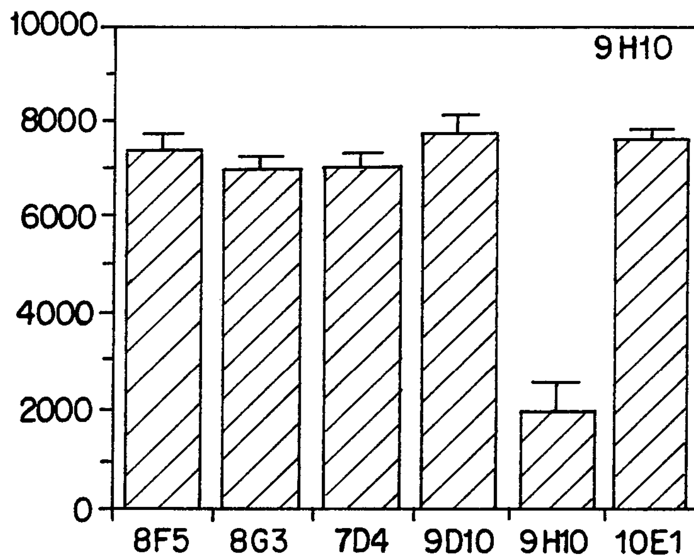

FIG. 1A

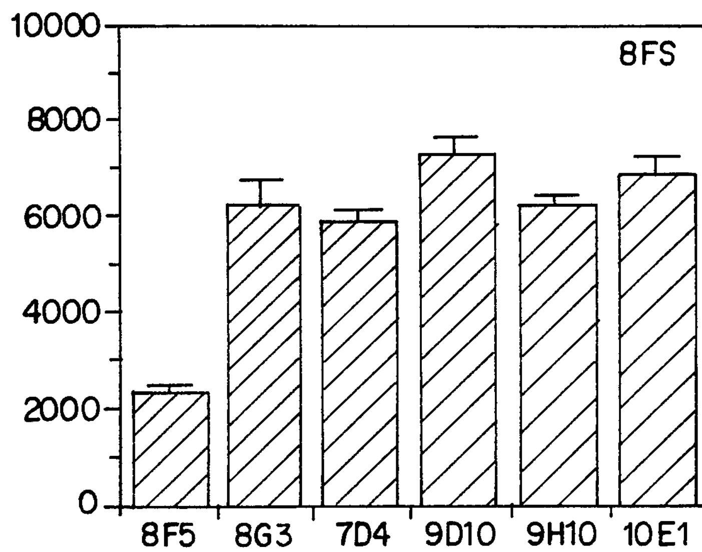

FIG. 1B

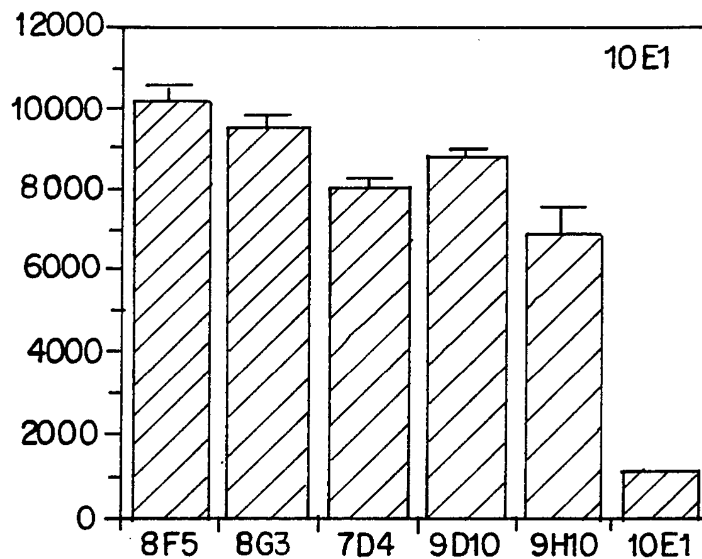

FIG.1C

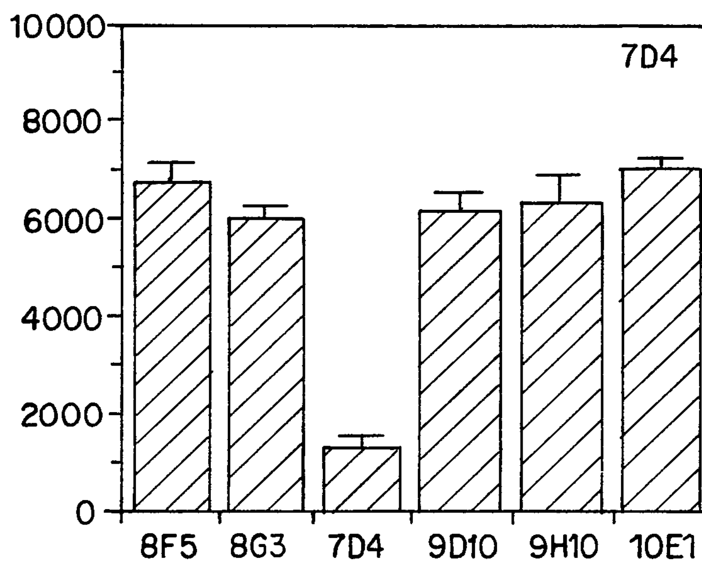

FIG. 1D

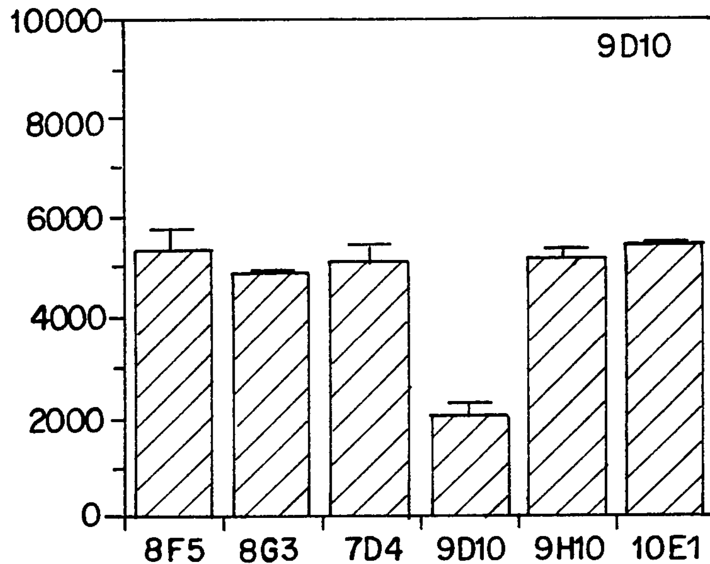

FIG. 1E

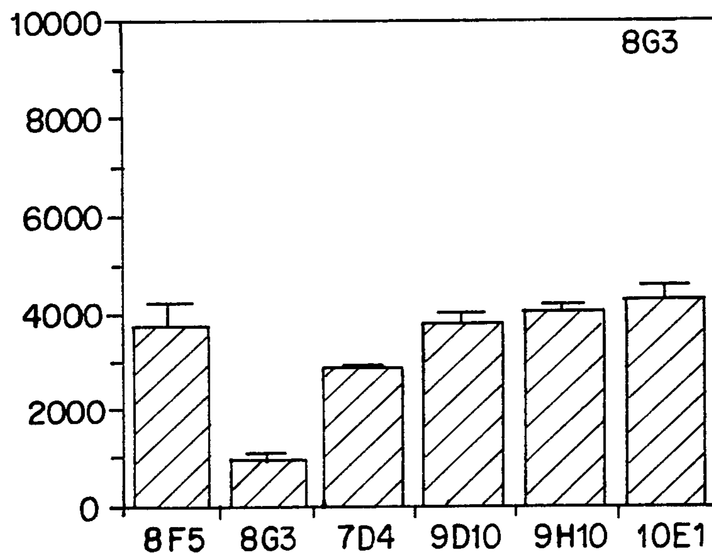

FIG. 1F

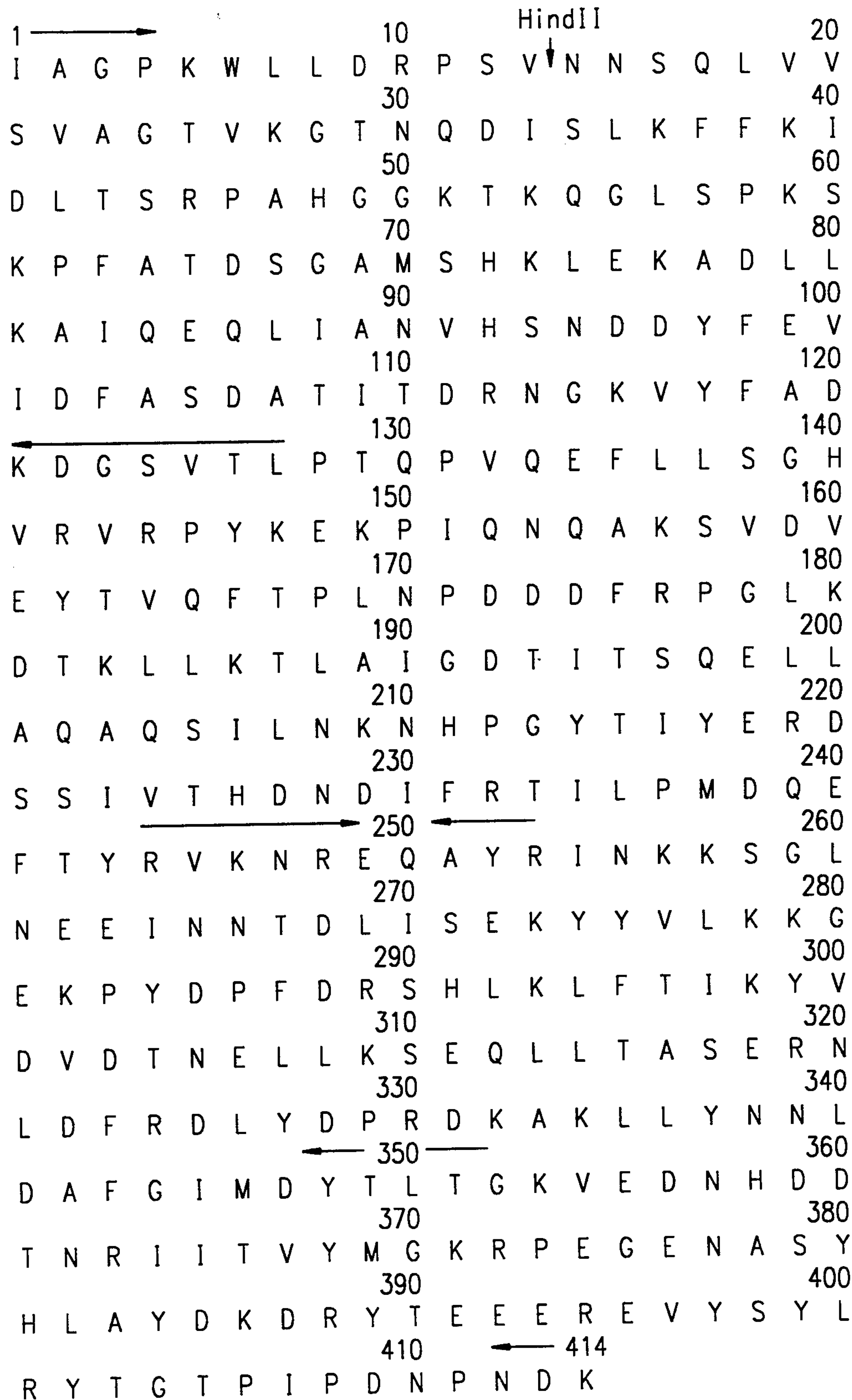

FIG.2A

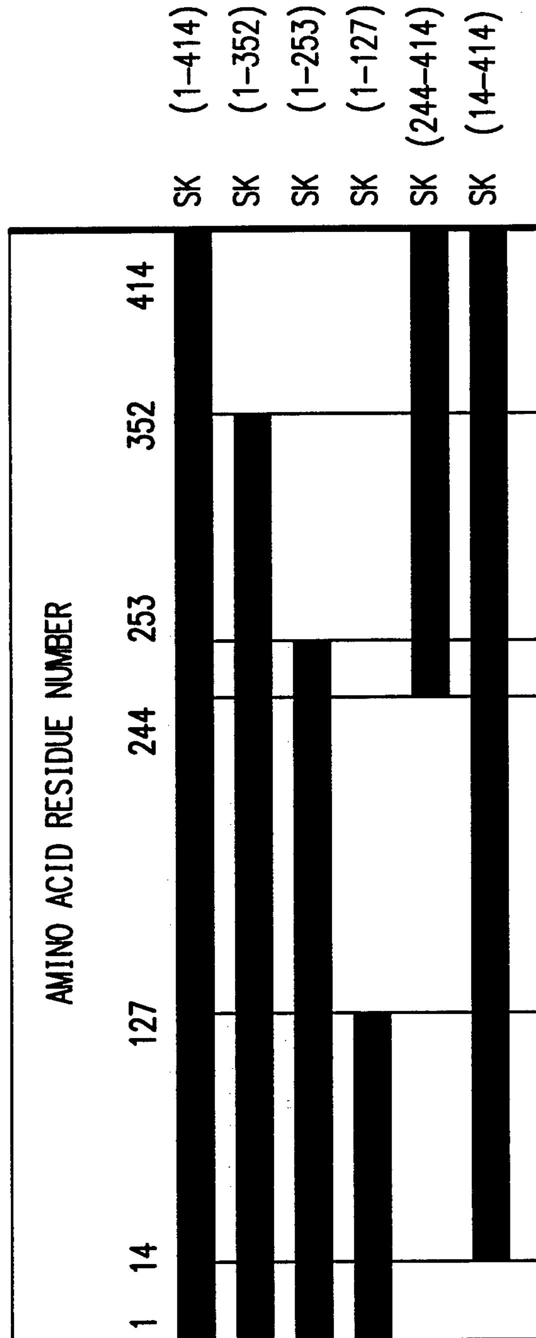

FIG.2B

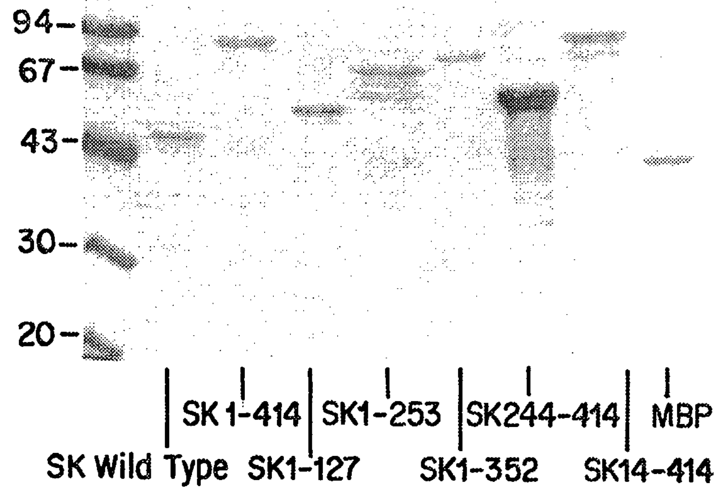

FIG. 3A

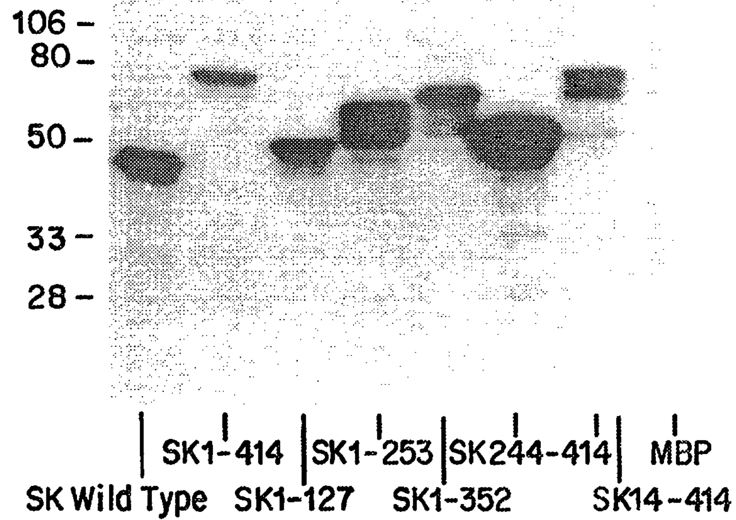

FIG. 3B

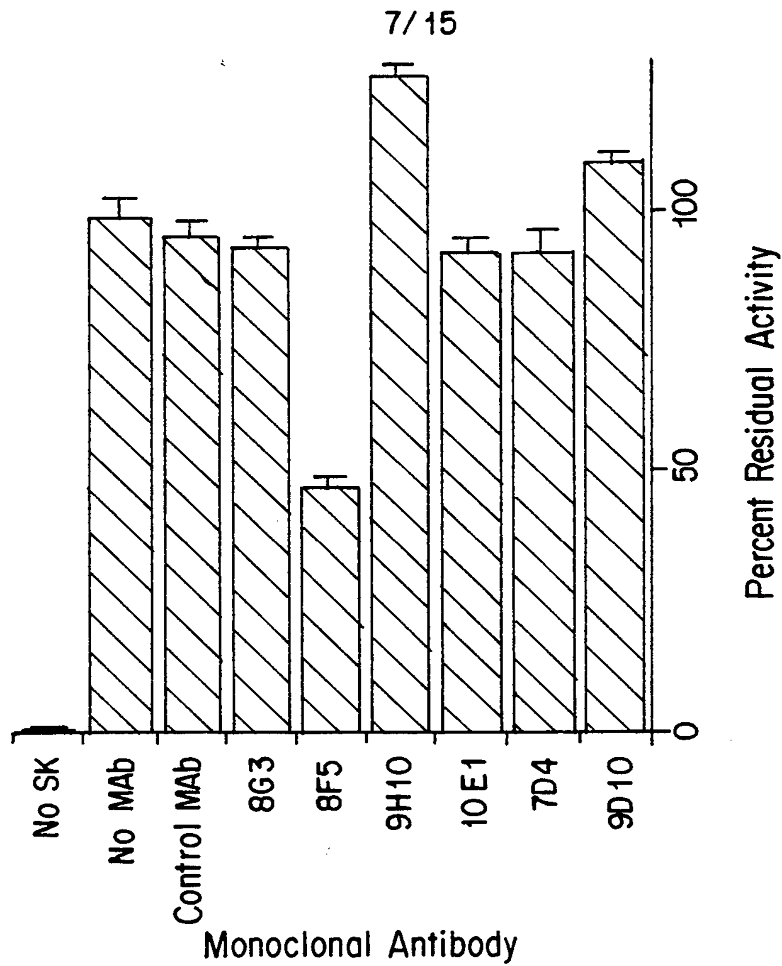

FIG. 4B

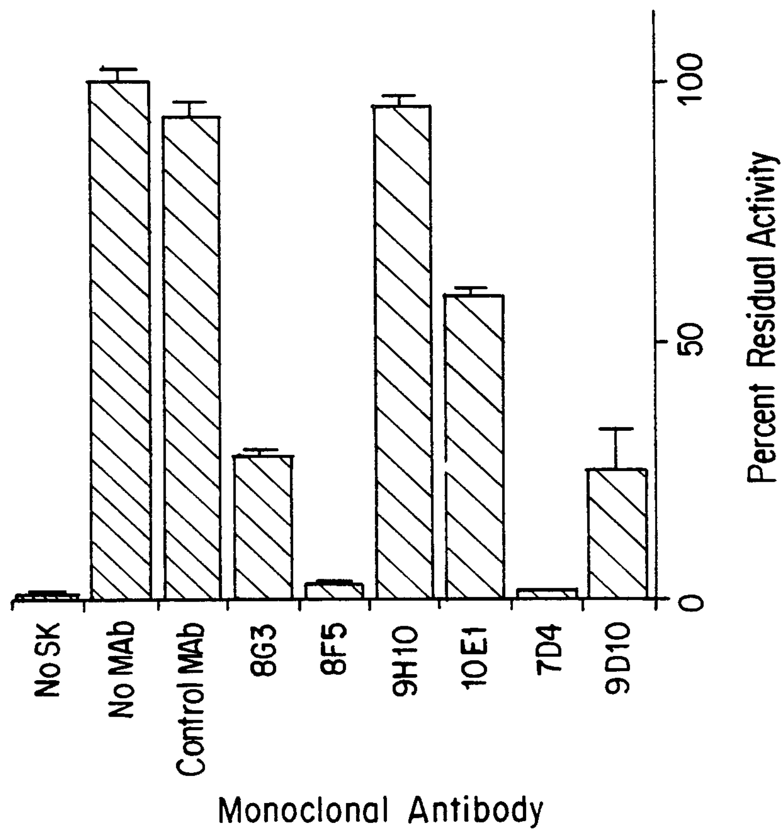

FIG. 4A

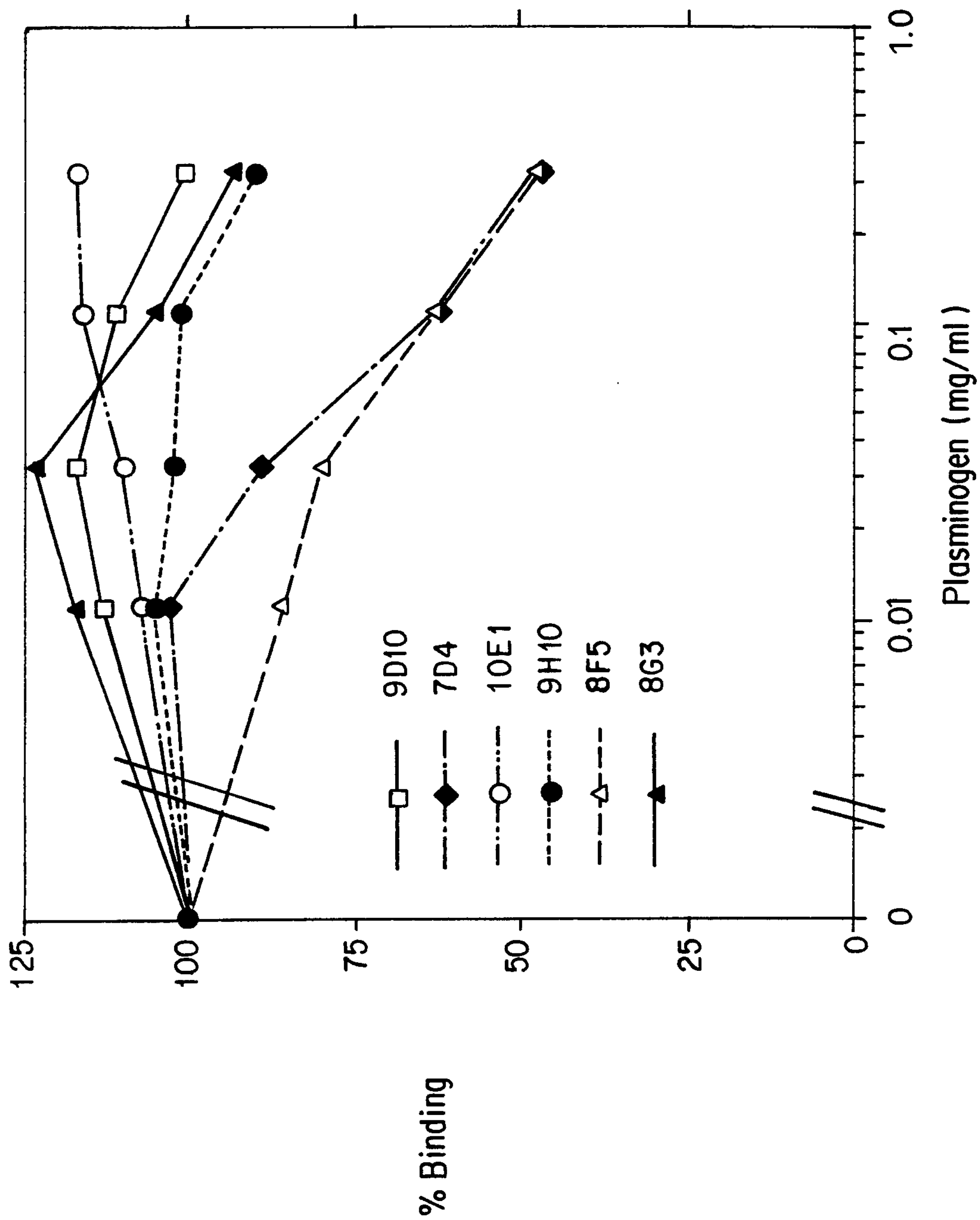

FIG.5

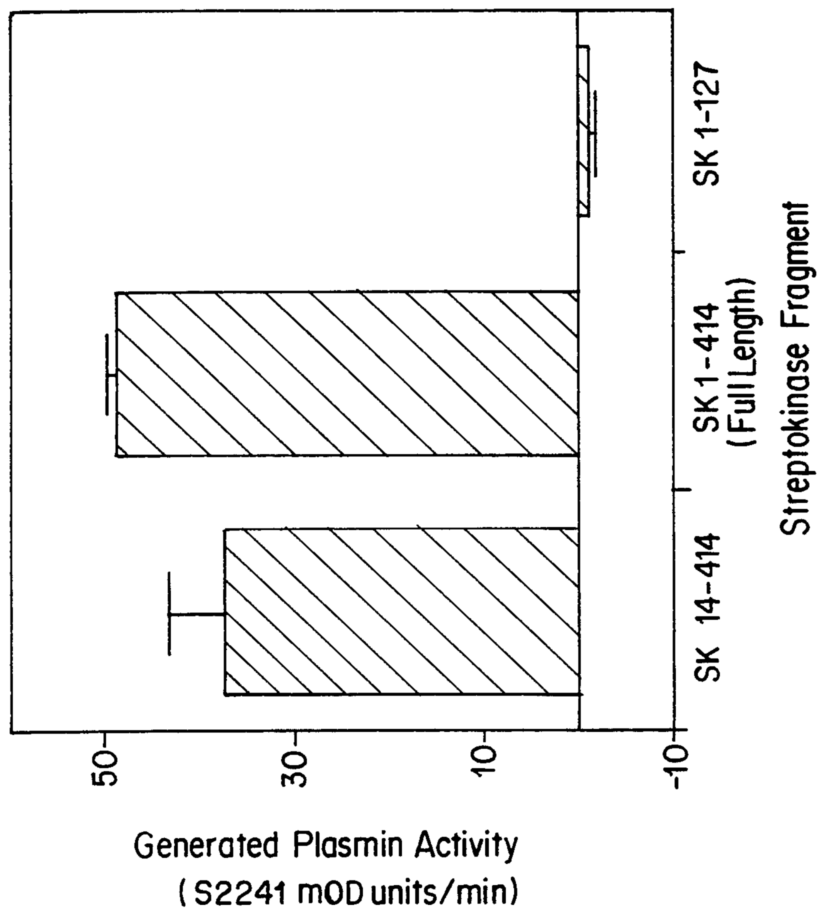

FIG. 6

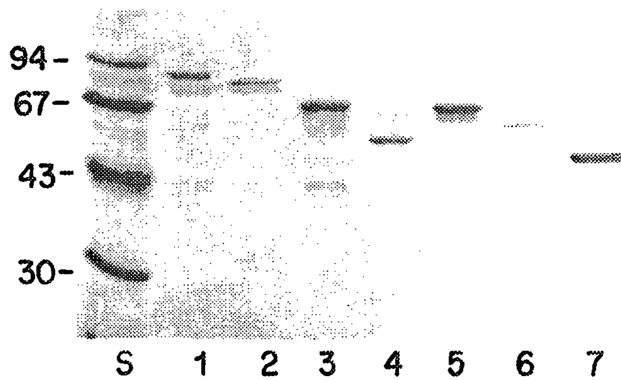

FIG. 7A

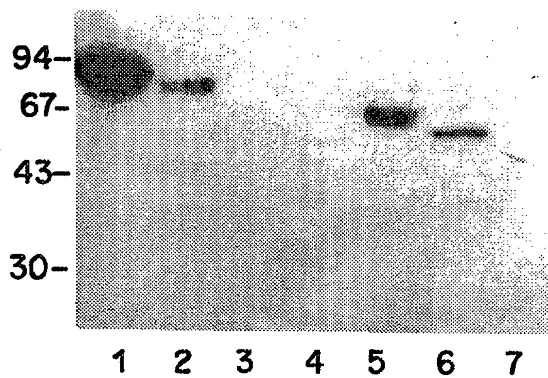

FIG. 7B

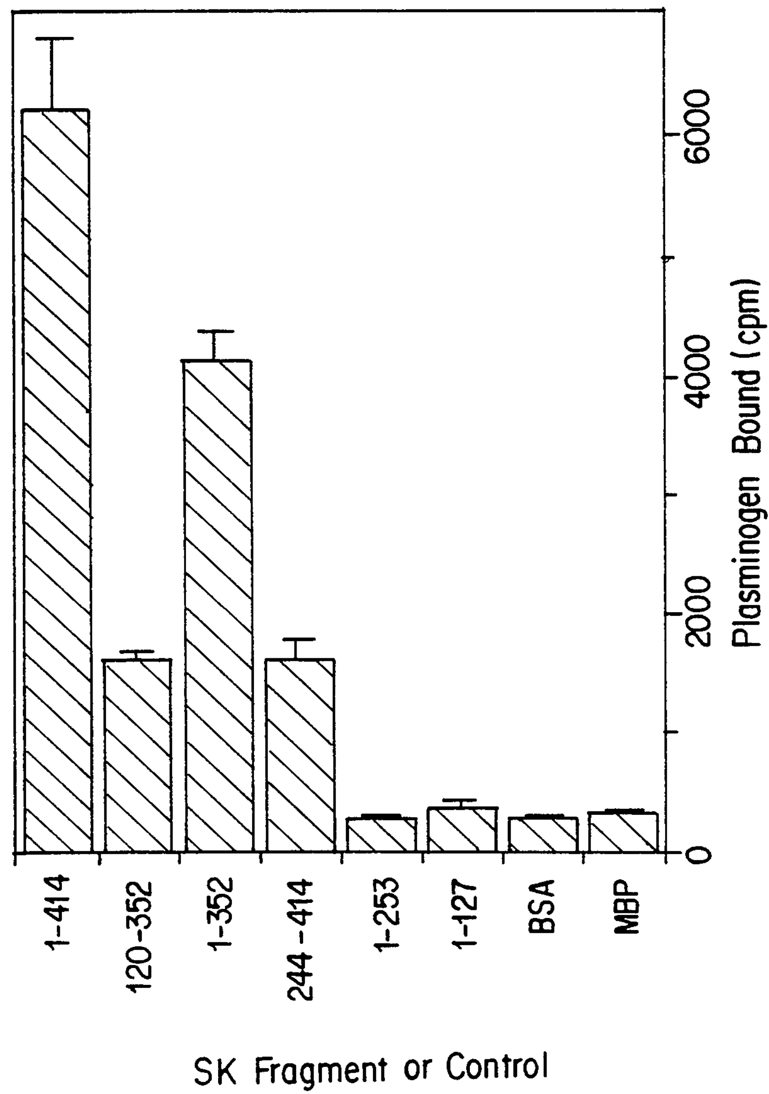

FIG. 8

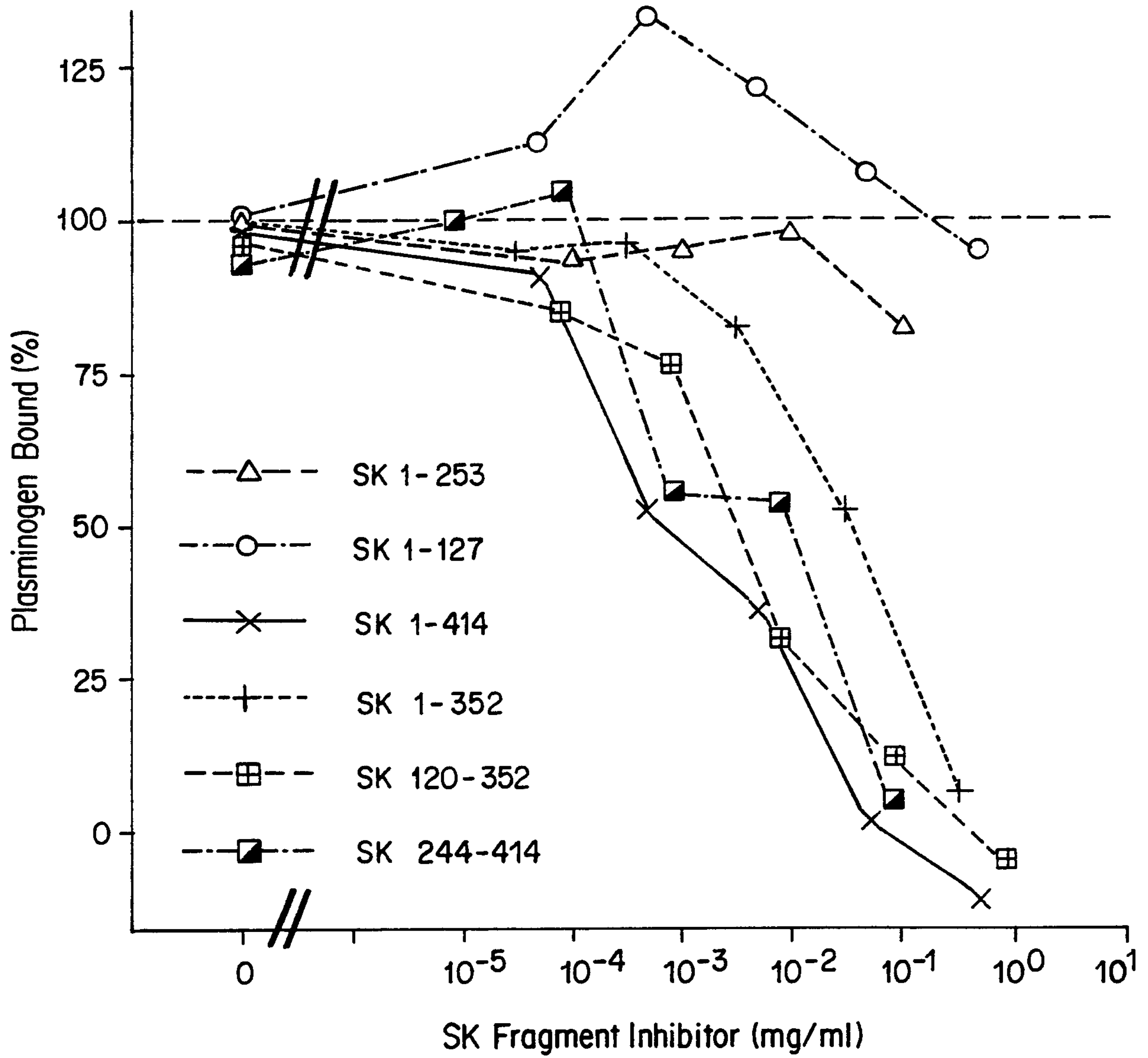

FIG. 9

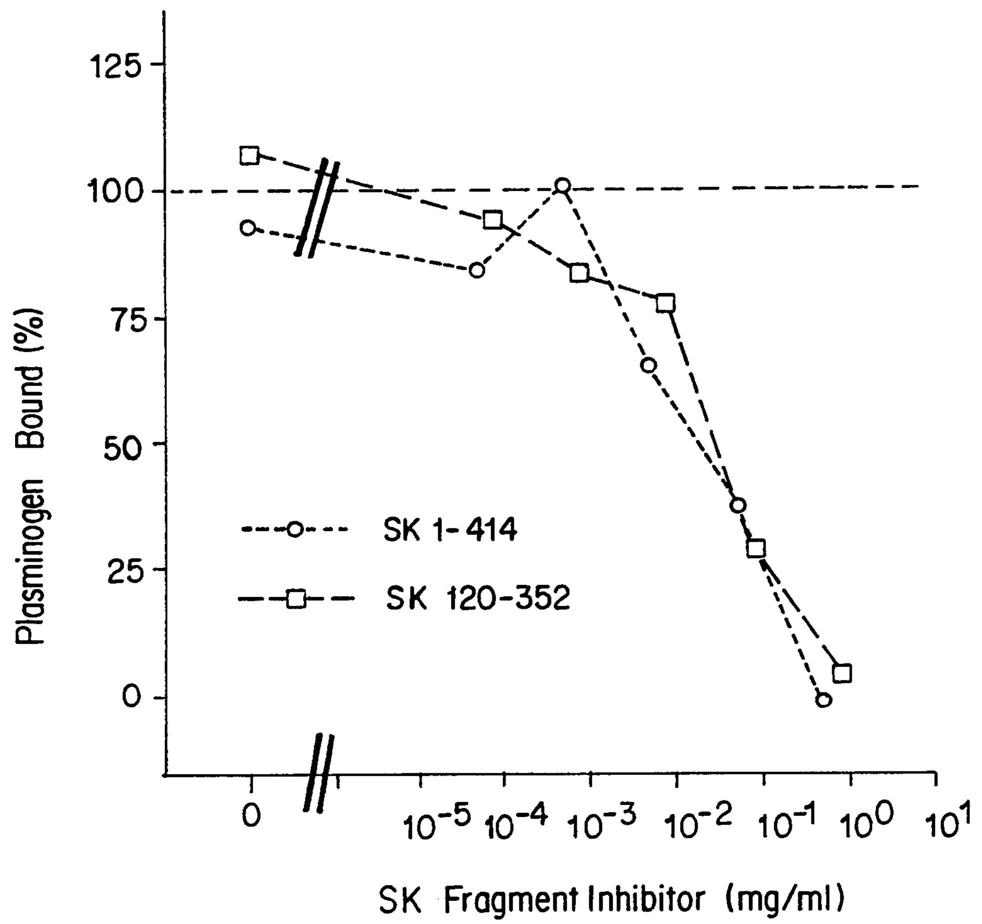

FIG. 10

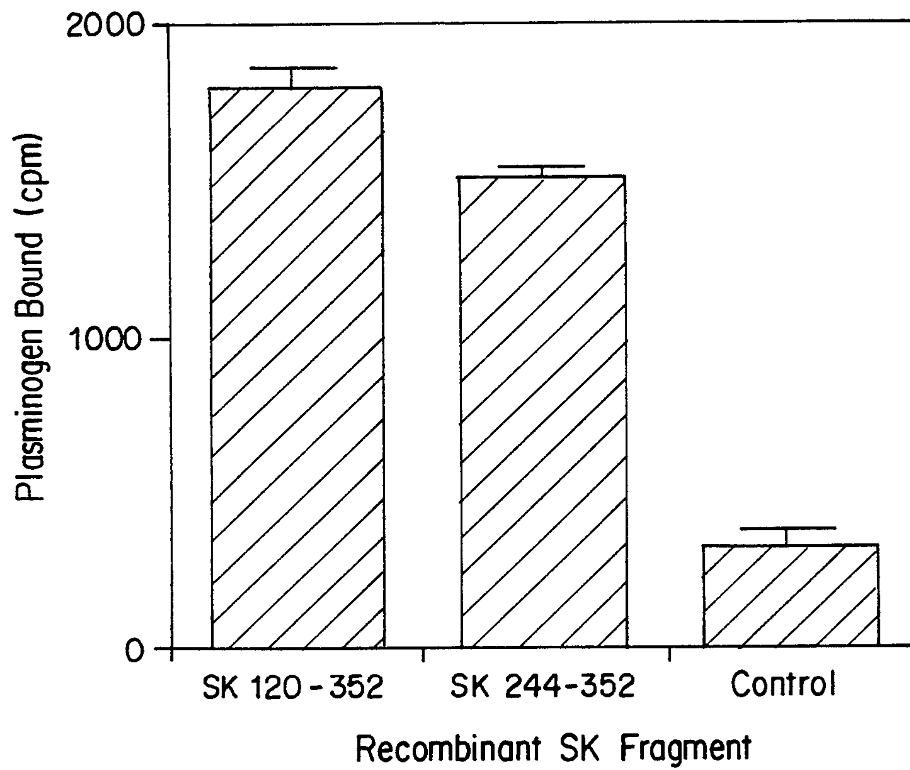

FIG. 11

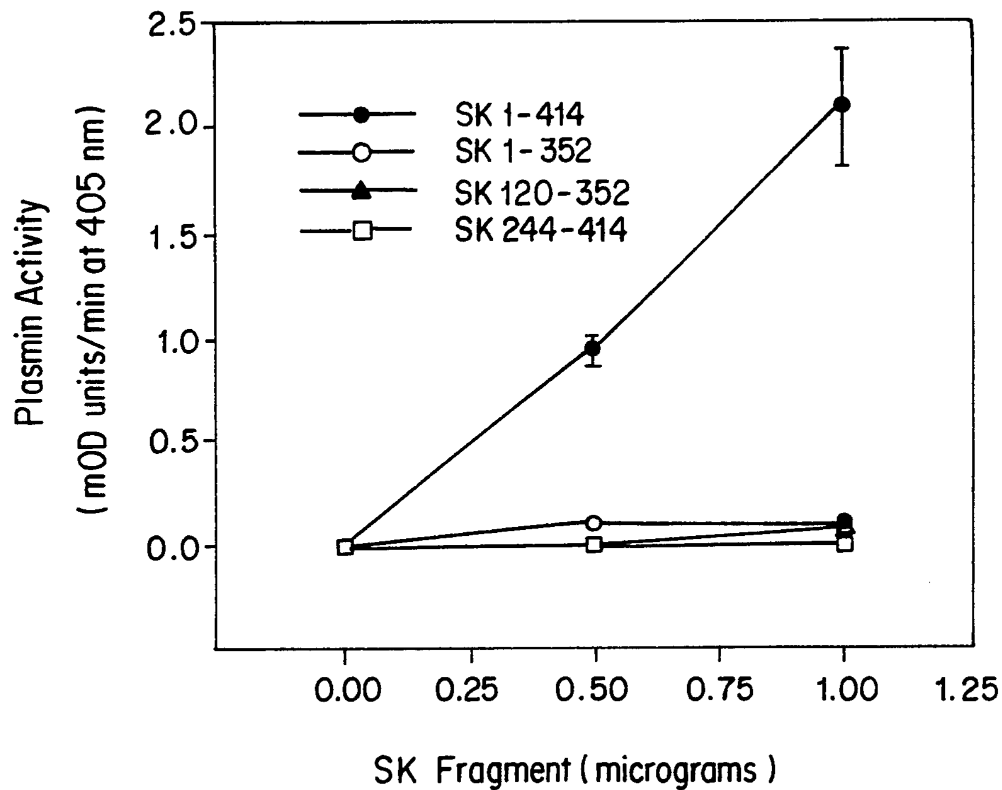

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09502

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.64; 435/216, 252.3, 320.1; 436/518; 514/12; 530/350, 388.26; 536/23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, PIR, SWISSPROT, GENBANK, UMBL, search terms: sequence, streptokinase, fragment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemistry, Volume 13, Number 10, issued 1974, Brockway et. al., "A characterization of native streptokinase and altered streptokinase isolated from a human plasminogen activator complex", pages 2063-2070, see page 2063.	1-21
X Y	DE, A, 137,325 (Losse et. al.) 29 August 1979, see pages 1 and 5.	13, 16 1-12, 14,15, 17-21
X Y	Gene, Volume 34, issued 1985, Malke et. al., "Nucleotide sequence of the streptokinase gene from Streptococcus equisimilis H46A", pages 357-362, see pages 358 and 360.	1, 4, 13, 16 2, 3, 5-12,14,15,17-21
X,P Y	US, A, 5,187,098 (Malke et. al.) 16 February 1993, see column 3, 5, 6, and 8.	13, 16 1,12,14,15, 17-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 December 1993	05 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KEITH FURMAN, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	The Journal of Biological Chemistry, Volume 251, Number 13, issued 10 July 1976, Siefring et. al., "Interaction of streptokinase with plasminogen: isolation and characterization of a streptokinase degradation product", pages 3913-3920, see pages 3913 and 3914.	13, 16,17 1-12,14,15, 18-21
X Y	British Heart Journal, Volume 66, issued 1991, Lynch et. al., "Immunoglobulin response to intravenous streptokinase in acute myocardial infarction", pages 139-142, see page 141.	13,16,20,21 1-12,14,15, 17-19
X Y	EP, A, 0,382,696 (Einarsson et. al.) 16 August 1990, see page 2.	13, 16 1-12,14,15, 17-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09502

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12N 1/00, 15/31,15/58; C07K 15/04, 15/28; G01N 33/53; A61K 37/02, 37/48, 37/547

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.64; 435/216, 252.3, 320.1; 436/518; 514/12; 530/350, 388.26; 536/23.7